DEC 4 1922

Medical Lib.

THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

VOLUME XXXVI, No. 6 DECEMBER 1, 1922

PUBLISHED MONTHLY

AT MOUNT ROYAL AND GUILFORD AVES., BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

COPYRIGHT, 1922, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Entered as second-class matter June 3, 1916, at the Post Office at Baltimore, Md., under the act of March 3 ,1897.
Acceptance for mailing at special rate of postage provided for in section 1103 ,act of October 3 ,1917.
Authorized June 29, 1918.

Made in the United States of America

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

The Journal of Experimental Medicine is designed to cover the field of experimental medicine. Information regarding contributions and subscriptions is given in full on the back cover.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

EDITED BY

STANLEY R. BENEDICT, New York, N. Y. LAFAYETTE B. MENDEL, New Haven, Conn.
H. D. DAKIN, Scarborough, N. Y. DONALD D. VAN SLYKE, New York, N. Y.

The Journal of Biological Chemistry is designed for the prompt publication of original investigations of a chemical nature in the biological sciences. It is issued monthly. Each volume consists of as many numbers as are required to make a total of approximately 800 pages. The subscription price is $5.00 per volume to subscribers in the United States and Canada, payable in advance; $5.25 to those in other countries. The number of volumes issued yearly averages four. The price of single copies varies; it is given on the back cover of each number. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Biological Chemistry*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

THE JOURNAL OF GENERAL PHYSIOLOGY

EDITED BY

JACQUES LOEB, New York, N. Y. W. J. V. OSTERHOUT, Cambridge, Mass.

The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter. It is issued bimonthly, one volume of about 600 pages appearing in a year. The subscription price per year (one volume) is $5.00, payable in advance. Single copies cost $1.00. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of General Physiology*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

STUDIES FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

The published results of investigations conducted in the Institute, or under its grants, are republished at irregular intervals in volumes designated *Studies from The Rockefeller Institute for Medical Research*. The subscription price is $2.00 per volume, payable in advance. A special rate of $1.00 per volume is made to those who subscribe to *The Journal of Experimental Medicine*, *The Journal of Biological Chemistry*, or *The Journal of General Physiology*. Remittances should be made by draft or check on New York, or by postal money order, payable to The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.

SEMIANNUAL LIST

The Institute publishes a *Semiannual List* of all papers by members of the staff and those working under grants from the Institute, stating the title and place of publication of these reports. The *Semiannual List* will be sent regularly upon application.

WAVERLY PRESS
WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

STUDIES ON ENDOTHELIAL REACTIONS.

VI. The Endothelial Response in Experimental Tuberculous Meningoencephalitis.

By NATHAN CHANDLER FOOT, M.D.

(*From the Department of Comparative Pathology, George Fabyan Foundation, Harvard Medical School, Boston.*)

Plates 54 to 56.

(Received for publication, July 3, 1922.)

INTRODUCTION.

As stated in the fourth paper of this series, it has been the writer's purpose to call attention to the importance of the vascular endothelium as a source of the mononuclear phagocytes, or macrophages, in conditions resulting from inflammation or injury. Three of the five preceding papers dealt with these phenomena in the case of tuberculous inflammation in rabbits—one with subcutaneous tubercles, one with pulmonary tuberculosis, and one with general miliary tuberculosis (Foot, 1920, *a*, *b*, 1921). In the present paper the reaction of the cerebral tissues to an infection with tubercle bacilli will be discussed. This subject has been reserved for special treatment because of that radical difference between cerebral and other tissues of the body, which might perhaps alter the type of reaction.

There is little doubt that tuberculous meningoencephalitis is a process practically limited to the meninges and their vascular extensions into the substance of the brain. This view appears to be accepted by those few who have written on the subject, upon which little work has been done during the past decade. It is difficult to find a detailed description of the histology of the cerebral lesions in this disease in most text-books on pathology, for the reason that they are to be considered usually a part of a general miliary tuberculosis. Cerebral tuberculosis has, however, some characteristics that differ

rather strikingly from the tuberculosis of other organs. It shows a well marked tendency to limit itself to the mesodermal elements of the brain, a far greater lymphocytic reaction, many large mononuclear cells circulating in the vessels of the affected area, and sometimes fewer giant cells are found here than in tubercles of other organs.

Perhaps the most detailed treatment of the process is to be found in Ranke's (1908) article, in which he gives an excellent historical sketch of the development of knowledge on this subject, tracing it from 1829 when it was first described by Abercrombie. Experimental work was not begun until Kure, working in Nissl's laboratory in 1898, produced tuberculous meningoencephalitis by drawing cotton threads impregnated with tubercle bacilli through the brains of dogs. This method elicited a mixed reaction in which there was, at first, a foreign body to be dealt with and later the infection resulting from the multiplication of the bacilli which had to be overcome. Delille improved on these experiments by injecting extracts of tubercle bacilli in ether, chloroform, and xylol. The tubercles produced in this way were found to be independent of the brain tissue, except where they encroached directly upon its substance.

Ranke's article concludes with a description of three cases of tuberculous meningitis with necropsy and a very careful report on the histopathological examination of the lesions, replete with morphological details based upon the study of preparations stained in a great variety of ways. In the course of this description he discusses large mononuclear cells of varying morphology that are first noted free in the blood of the pial vessels. These cells were first discussed by Tigges in 1863 and interpreted as exfoliated endothelial cells by Sawada in 1901, Diamond taking a similar view as to their origin at about the same time. Ranke, after describing them exhaustively and with the greatest minuteness of detail, leaves the question of their origin open for further investigation and future decision. This is particularly interesting as Farrar (1908), in an article published in the same volume and issued from the same laboratory, describes similar cells in connection with experiments on the reaction to elder pith discs inserted into the cerebral cortex of rabbits, ascribing their origin to the pial endothelium and also tracing the grid cells from the vascular endothelium of the meninges.

Gehry (1909), writing in the following year, takes up the question of the origin of these macrophages and, after discussing them at length, categorically states that they are derivatives of the fixed tissues. As this term is loosely employed in the German literature, it may mean the vascular endothelium, the adventitial connective tissue, or the mesothelium. All these writers are agreed that the brain tissue proper does not respond to foreign bodies, nor to tuberculous inflammation, except secondarily—when it is invaded by the agent in question and by the mesodermal reaction to its presence. In such areas the nerve cells die off, to be replaced by the multiplication of their satellite glia cells, which act as mere stopgaps. The glia cells form clusters, or nests, and multiply usually by amitotic

division, although mitotic figures are sometimes found. Rod cells are found in the neighborhood of tuberculous lesions in man; the matter of their origin is fully discussed by Uyematsu (1920) in an article on diffuse cerebral sclerosis. He believes them to be modified glia cells, while Alzheimer and Nissl have considered them to be endothelial in origin. Farrar (1908) states that they do not occur in the rabbit's brain.

Shorter discussions of tuberculous meningitis will be found in the section on vascular lesions of the brain and cord by Nonne and Luce (1904) in the Handbuch der pathologischen Anatomie des Nervensystems. The fact that cerebral tuberculosis is taken up under the heading of vascular diseases speaks for itself. Lugaro, in the same text-book, gives a good description of the glia cell and its manifold variations in type. According to him, it is the phagocyte of the ectodermal brain tissue, usually multiplying by direct division; it may form giant cells, spider cells or astrocytes, and may also phagocytose and replace ganglion cells, etc. In recent years an article by Friedenwald and Greenfeld (1915) brings the subject of cerebral tuberculosis more nearly to date—but their description of the pathological histology of the lesions is rather meager.

From this review it will be seen that the main question left for decision is the origin of the mononuclear macrophages that circulate in the meningeal vessels and through the lymph spaces of the pia-arachnoid in tuberculous meningitis. That the tubercles are formed in, or near vessels and that their epithelioid cells are probably of endothelial origin has been fairly well established. In order to decide this point, if possible, the following experiment was performed.

EXPERIMENTAL.

Five adult rabbits were anesthetized in turn and a 0.5 cm. opening trephined in the right parietal bone at a point midway between the coronal and lambdoid sutures, about 1 cm. to the right of the sagittal suture. A few drops of a suspension containing in all 0.2 mg. of a 1 month culture of bovine tubercle bacilli on glycerol agar were then injected into the right hemisphere through a 24 gauge needle 1 cm. in length. The same strain of bacilli used in the preceding experiments of the series was employed here. The suspension was prepared as before; a weighed amount of bacilli was rubbed up in sterile salt solution and, after thorough shaking, diluted until 1 cc. equalled 1 mg. of bacteria. Of this suspension 0.2 cc. was injected through the needle, the latter being held as nearly vertical as possible, and

inserted to its full length, releasing the bacilli at the same depth in each brain. There was some leakage from the needle stab after withdrawing the instrument, but this was not without its advantages. The wound was then closed without replacing the bone disc.

All the rabbits were given 10 cc. of 1 per cent aqueous Niagara blue (2 B recrystallized), intraperitoneally, and 5 cc. of Higgins' waterproof drawing ink and distilled water, 50 per cent, intravenously. These dyes were administered thereafter twice a week, until the rabbits became too sick for further injections.

One rabbit became sluggish and lost its appetite at the end of the 2nd week and was killed on the 14th day by injecting Zenker's solution into the right common carotid, under full anesthesia. Nothing was found on gross examination at necropsy, but the use of Niagara blue produces such an intense bluing of the tissues that an early general miliary tuberculosis was overlooked. The other four rabbits became very sick 5 days later, the 19th of the experiment; all were weak and apathetic and all but one had temperatures of 102–102.6°F. by rectum, that one having but 99.9°. The next day the rabbit with the lowest temperature was found dead, after paralysis in its fore legs. It showed an early general miliary tuberculosis and the lower lobe of the right lung was almost completely consolidated and covered with a fibrinous exudate, later ascertained to be an almost complete infarction. The brain showed suspicious sandy nodules about the site of injection of the tubercle bacilli.

The third rabbit was killed the same day, by injecting Zenker's solution as before. It had a definite right-sided weakness for 2 or 3 days preceding death. The brain showed a well developed basal tuberculous meningitis and extensive tuberculous involvement of the brain substance around the injection wound, with edema and swelling of the right hemisphere and right choroid plexus. This was the only brain that showed much gross staining with the Niagara blue, which was confined to the meninges. The internal organs, although they showed a miliary tuberculosis under the microscope, were grossly negative.

The fourth rabbit became progressively weaker, and developed a somewhat inconspicuous left-sided paresis. On the 23rd day of the experiment it was killed in the same manner as were the others; necropsy showed basal meningeal tuberculosis, tubercles along the needle wound, and general miliary tuberculosis.

The fifth rabbit, a strong buck, developed a tendency to turn to the left on the 21st day. After becoming very weak it was killed on the 26th day of the experiment, by an injection of Zenker's fluid into the right common carotid, under ether. At necropsy the findings were similar to those of the preceding four, except that there was more edema of the right temporal lobe near the base.

It is interesting to note the failure of the blue stain to take in any of the brains, with one exception, in which the meninges were distinctly blue. The lesions were not grossly dyed.

In all cases the brain, liver, lungs, spleen, and kidneys were hardened in Zenker's fluid, embedded in paraffin, and cut to 5 microns. Sections were stained with eosin-methylene blue, Mayer's aqueous carmalum, phosphotungstic acid-hematoxylin, and Ziehl-Neelson carbolfuchsin, with Delafield's hematoxylin as a counterstain.

Microscopic Examination.

Each rabbit shows a tuberculous meningitis, the interlobar meninges and choroid plexus are also involved, the vessels running into the brain from the meninges are surrounded near their origin by tubercles and, deeper in the cortex, by a dense zone of lymphocytes and plasma cells. The vascular lesions are more marked in some animals than in others, according to the severity of the infection. The needle puncture is filled with masses of debris, polymorphonuclear leucocytes, and caseous material, surrounded by a zone of actively proliferating endothelial cells in close proximity to one another, which penetrate the surrounding nerve tissue in the form of long, ray-like extensions. Outside of this tuberculous tissue is a zone of degenerated nerve tissue, in which one finds edema, many polymorphonuclears and altered glia cells, and the remnants of nerve cells, but no ink-bearing endothelial macrophages.

In general all the tubercles are more or less deeply pigmented with ink granules; there is little or no Niagara blue, except in the form of coarse granules in the discrete macrophages of the meningeal lymphatics, cells which also contain ink. The changes in the brain tissue are typical and conform to those discussed in the Introduction: degeneration of nerve cells, increase in the number of glia cells, without evidence of mitosis, and polymorphonuclear infiltration of the most affected regions. There are often glia cell nests, or nebulæ; the satellite, or *trabant* cells fill the spaces left by the death of the ganglion cells. Apparently the glia cells perform those functions that are elsewhere effected by the endothelial cells, for ink-bearing macrophages are not found at any distance from the tubercles.

The free mononuclear cells discussed so extensively by Ranke and Gehry are found in large numbers in the veins and capillaries of the meninges, less frequently in the arteries. Fully a third to a half of their number contain ink granules, which indicates their endothelial

origin. These cells are frequently found in mitosis while free in the lumen of the blood vessel; three such figures were encountered in the course of half an hour's search, while counting ink-bearing macrophages (Fig. 1).

There is no need for a detailed description of the lesions; that they contain ink, wherever they may be situated, is the main fact to emphasize; hence they are composed chiefly of cells of endothelial origin (Figs. 2 and 3). The reasons for considering any ink-bearing cell as endothelial already have been discussed sufficiently in the preceding papers. Thus the cerebral tubercle conforms in its histogenesis to tuberculosis of other organs. The more interesting point for consideration is the question as to the locus of origin of these endothelial cells. In this case the local activity of the capillary endothelium in the neighborhood of the lesions is not nearly as marked as it was in the lesions of other organs studied in the preceding experiments. Thickening of the capillary endothelium and an increase in the number of its nuclei are evident here and there, especially where the brain tissue has been more extensively destroyed, but on the whole these changes are not at all marked, nor are mitoses found to occur in the sessile endothelial cells of these vessels. It will be recalled that these lining cells were frequently found in mitosis in the other experiments and that this usually occurred near the lesions.

Ink is not found in the ependymal cells of the ventricles or choroid plexus (Fig. 4). Where tubercles occur, they are always beneath the ependyma and covered by it, and they contain the usual amount of ink. The ependymal cells are, therefore, very similar, in their lack of response to the tubercle bacilli and injected pigments, to the mesodermal cells of the peritoneum. It was always the subperitoneal, perivascular tissue that responded in the cases of tuberculous peritonitis studied in the previous experiments.

Whence, then, come the endothelial cells that go to form the numerous tubercles in these brains? Ranke and Gehry called attention to circulating mononuclears. These cells are very abundant in all five of the rabbits studied; furthermore, many of them are fixed in the act of mitosis, proving that they are actively proliferating. Much has been written about them, little definitely proved; they probably come from other organs more richly supplied with capillaries than

is the brain. Mallory has long maintained the endothelial origin of circulating mononuclears and more recently McJunkin (1919) has reached a similar conclusion. Downey (1916–17), Evans (1915), and Simpson (1922) suggest that they proceed largely from the reticuloendothelium of the hematopoietic organs, particularly the spleen. A glance at the sections from the spleen (Fig. 5) and the liver (Fig. 6) of the animals of this experimental series is sufficient to make it clear that pigmented endothelial cells are leaving the capillary walls and entering the veins, to be thrown into the circulation in numbers somewhat larger than would be the case following the mere injection of ink (Simpson's "shower of macrophages"). In the lung they are also found in the larger vessels, and the pulmonary endothelium is rather swollen and active in places, but it must be remembered that here, as well as in the other organs, an active miliary tuberculosis is in progress. Although not much increased in number over what one finds in control animals without infection, they show a process not seen in controls—the phenomenon of mitotic division while free in the blood stream.

Supplementary Experiment.—In order to rule out at least one of the hematopoietic organs as the sole source of these circulating macrophages, the spleen was removed from four rabbits, which were then inoculated with tubercle bacilli of the same strain and in the same manner as the preceding series of animals. The cultures were younger (2 weeks) so that a smaller dose was administered, in order to decrease the toxicity. Only 0.16 mg. was given in these cases. Following the operation, which was performed under ether anesthesia, 5 cc. of Higgins' ink and distilled water in equal parts were injected into an ear vein 1, 5, and 8 days afterwards; the dose was repeated in two of the rabbits on the 12th day. These two died almost immediately with symptoms suggesting cerebral embolism. Necropsy showed no gross lesions, beyond an unusually light-colored liver and almost jet-black lungs. Microscopic examination showed that death was due to cerebral emboli of ink, which were scattered all through the precapillaries of the cortex and were plugged quite solid with the ink. There were occasional ink emboli in the lungs, but not sufficient to give death from this cause. The remaining two rabbits went uninjected for 2 weeks, when another intravenous administration of 5 cc. of the

ink mixture was given, this time uneventfully. Another injection, 1 week later, killed the third rabbit in precisely the same way the other two had been overwhelmed and necropsy gave similar findings. The fourth rabbit was allowed to go uninjected for 4 weeks and was then killed by introducing Zenker's fluid into the right common carotid under anesthesia.

The glass pestle used in triturating the bacilli for the suspension injected into these splenectomized animals apparently was not sufficiently cooled after flaming, for the bacilli were killed. Very good local reactions (Hodenpyl tubercles) were, however, obtained; they differed from those of the preceding experiment merely in so far as they were localized, did not spread to other organs, and were not accompanied by generalized symptoms. Despite the fact that the spleen had been removed, the tubercles were formed and the macrophages were found in apparently undiminished numbers lying free in the meningeal vessels. An examination of the liver and bone marrow revealed no increased production of macrophages, indeed they seemed to contain less ink than in the case of control animals, and there was no undue activity of their endothelial elements. The lungs, on the other hand, showed changes so striking that they call for another experiment to determine their cause and to follow the course of their development. This will be described in another article. The proliferation of the pulmonary endothelium, the swelling of its component cells, and the increased amount of contained ink in the organ are most remarkable.

We may conclude, then, that cerebral tubercles are produced somewhat differently from those of other organs, in that the endothelial cells that form them are evidently derived only in part from the local capillaries; the circulating macrophages showing much more proliferative activity than the sessile endothelial cells of these capillaries probably form a more important source of supply. Hence it appears that a much greater proportion of the endothelial cells of cerebral tubercles is derived from the circulating blood than is the case in tubercles of other organs. This conforms with the older French theories on the histogenesis of the tubercle.

CONCLUSIONS.

1. Experimental cerebral and meningeal tubercles in the rabbit are formed from cells of endothelial origin.

2. These cells are derived apparently from other sources than the neighboring capillary endothelium alone.

3. The circulating macrophages, which, in this case, are capable of multiplying by mitosis while still free in the blood, are drawn upon in the formation of the cerebral tubercle.

4. Splenectomy has not materially decreased the available supply of circulating macrophages in this experiment.

5. While these cells may originate in the endothelium of the liver and bone marrow, the lung appears to play a much more important rôle in this respect than has been hitherto suspected.

BIBLIOGRAPHY.

Downey, H., *Anat. Rec.*, 1916–17, xi, 350.
Evans, H. M., *Am. J. Physiol.*, 1915, xxxvii, 243.
Farrar, C. B., *Hist. u. histopath. Arb. Grosshirnrinde*, 1908, ii, 1.
Foot, N. C., *J. Exp. Med.*, 1920, *a*, xxxii, 513.
Foot, N. C., *J. Exp. Med.*, 1920, *b*, xxxii, 533.
Foot, N. C., *J. Exp. Med.*, 1921, xxxiii, 271.
Friedenwald, E. B., and Greenfeld, W., *Am. J. Dis. Child.*, 1915, ix, 508.
Gehry, K., *Arch. Psychiat.*, 1909, xlv, 59.
Lugaro, E., in Flatau, E., Jacobsohn, L., and Minor, L., Handbuch der pathologischen Anatomie des Nervensystems, Berlin, 1904, i, 189.
Mallory, F. B., The principles of pathologic histology, Philadelphia and London, 1918.
McJunkin, F. A., *Am. J. Anat.*, 1919, xxv, 27.
Nonne, M., and Luce, H. M., in Flatau, E., Jacobsohn, L., and Minor, L., Handbuch der pathologischen Anatomie des Nervensystems, Berlin, 1904, i, 200.
Ranke, O., *Hist. u. histopath. Arb. Grosshirnrinde*, 1908, ii, 252.
Simpson, M. E., *J. Med. Research*, 1922, xliii, 77.
Uyematsu, S., *J. Nerv. and Ment. Dis.*, 1920, li, 514.

EXPLANATION OF PLATES.

PLATE 54.

FIG. 1. Macrophages circulating in a meningeal vessel. Some are clumped, others discrete; one of these cells is seen in mitosis at the center of the photograph. × 400.

FIG. 2. Cerebral tubercle. The ink granules are so coarse that most of them are readily confused with nuclear material in the photograph. Several of the epithelioid cells near the periphery show finer granules. × 335.

PLATE 55.

FIG. 3. A meningeal tubercle. The ink shows to better advantage. × 335.

FIG. 4. Tubercle in the choroid plexus. Note that carbon is not seen in the ependymal layer, which remains aloof from the process going on beneath it. × 335.

PLATE 56.

FIG. 5. Group of macrophages leaving the spleen, one of them in mitosis, most of them heavily laden with ink. × 335.

FIG. 6. Macrophages in an hepatic vein and in the sinusoids of the liver, in one place clumped into a mass. × 335.

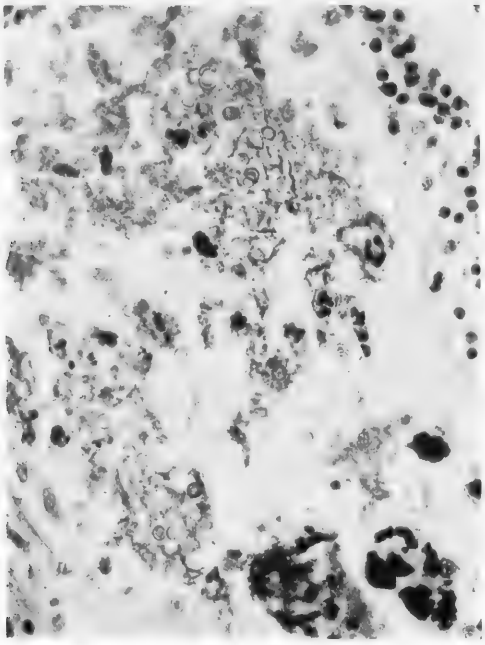


FIG. 1.

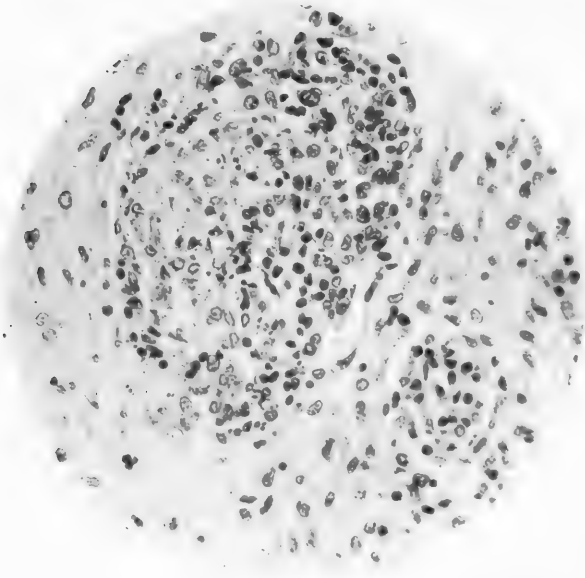


FIG. 2.

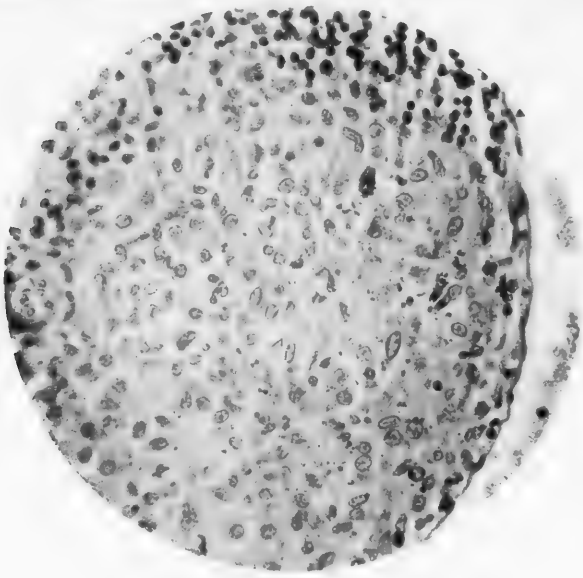


FIG. 3.

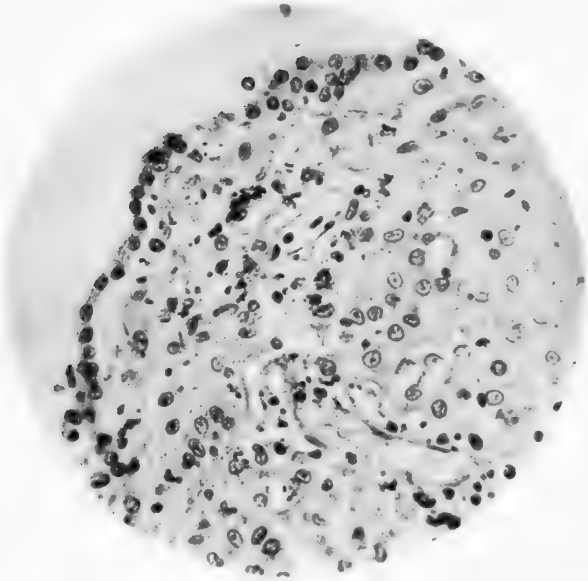


FIG. 4.

(Foot: Endothelial reactions. VI.)

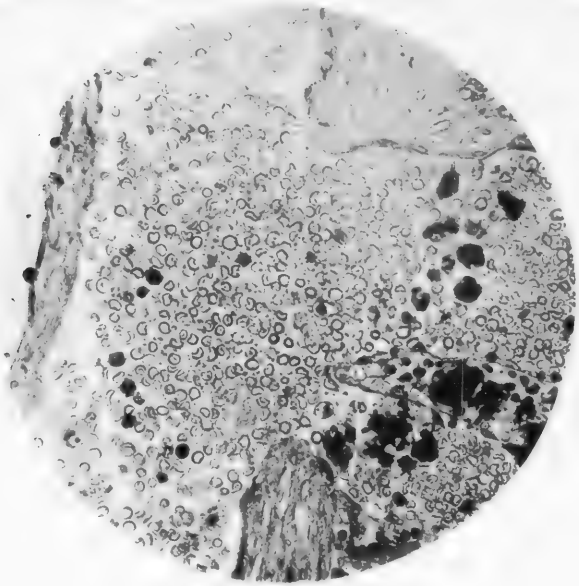


FIG. 5.

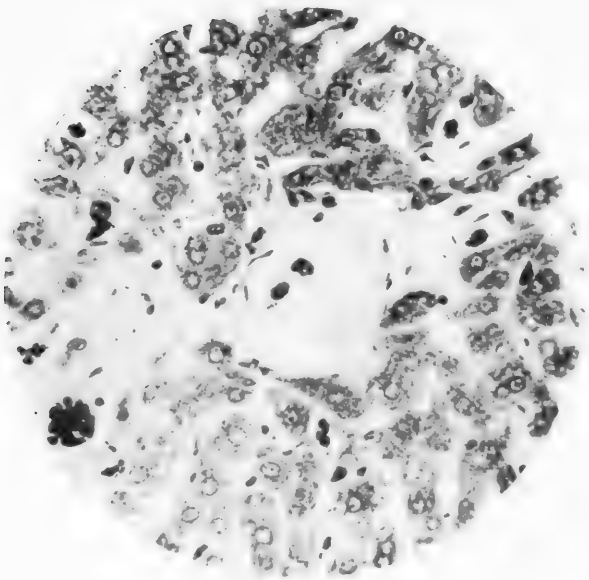


FIG. 6.

(Foot: Endothelial reactions. VI.)

SPIROCHÆTA EURYGYRATA.

A Note on Its Life History and Cultivation.

By MARY JANE HOGUE, Ph.D.

(From the Department of Pathology and Bacteriology of the Johns Hopkins University, Baltimore.)

Plate 57.

(Received for publication, June 24, 1922.)

For some time it has been known that spirochetes are occasionally present in the intestine of man.

In 1894 Kowalski (1) mentioned finding large numbers of spirochetes in the stools of patients suffering with cholera, and said he had seen them in the cholera epidemic in Paris in 1884. In 1903 Le Dantec (2) found almost pure cultures of spirochetes in the dysenteric stools of a patient in Southwest France. He believed them to be the cause of the dysentery. In 1907 Mühlens (3) reported finding small spirochetes with two to four spirals in a case of amebic dysentery, in Southwest Africa. He described them as active and as having pointed ends. He found similar organisms in children suffering with summer diarrhea and also in cholera stools. He considers them non-pathogenic.

Werner (4) in 1909 described two types of spirochetes which he found in his own stool after typhoid fever: one which he calls *Spirochæta eurygyrata*, loosely coiled, very active, and flexible, with rarely more than two spirals, mostly an S form; the other type, *Spirochæta stenogyrata*, tightly coiled, not so active, and less flexible. He finds it hard to compare these types with Le Dantec's spirochetes on account of the latter's meager description, but concludes that his first type corresponds to Le Dantec's. He found Type I in an Arab who had no intestinal disturbances, and also in a case of chronic diarrhea, and found both types in three cases of sprue. He concludes that it is impossible to say whether or not the spirochetes are pathogenic.

In 1914 Thomson and Thomson (5) also reported finding spirochetes in the alimentary tract of man and thought they corresponded to Le Dantec's. These measured 8 to 20 μ. They had rather blunt ends, large irregular curves, and showed a great flexibility of the body.

In 1916 army medical officers began reporting the presence of *Spirochæta eurygyrata* as an intestinal parasite. Fantham (6) described it as having pointed

ends, and being 3 to 15 μ in length. He believed that the number of coils is not a specific character, but is variable, depending on the thickness of the organism. Thomson and Thomson (7) list seven varieties of spirochetes frequently found in feces, especially in diarrheal condition. Macfie (8), at autopsies, showed spirochetes to be present in the stomach, at various parts of the small intestine, in the cecum, and in the large intestine. They appeared to him to be non-pathogenic, but he concludes that this question cannot be considered settled.

Luger (9) found *Spirochæta eurygyrata* in thin, often bloody, stools. He believed that the spirochetes can appear in varying numbers in the intestine, as normal parasites, but under certain conditions, not well understood, they may lead to a serious injury of the intestine, the therapy of which is little known. In 1918 Porter (10) described them as being 15 μ long with tapering ends. She believed that their presence in large numbers is indicative of intestinal disorder. Recently Langendörfer and Peters (11) described the case of a soldier who had a spirochetal infection accompanied by bloody stools. The spirochetes had six to eight tight spirals. They were motile, but not flexible. The Wassermann reaction was negative. The patient was treated with salvarsan, and in 3 months was well. These authors were not sure that the spirochetes were the cause of the dysentery, but were certain that they were present and caused the bloody condition.

It will be seen from the foregoing résumé that there is a great variation in the descriptions by different writers of the organisms which many of them call *Spirochæta eurygyrata*, and also in their conception of its pathogenicity. It was with the hope of reconciling some of these rather contradictory reports that the present work was begun.

The material from which this study has been made was sent to the laboratory, January 26, 1921. Loopfuls of the liquid feces, containing *Spirochæta eurygyrata*, were transferred to culture media and the organism was cultivated. On March 2, two pure lines, Strains S1 and S2, were isolated. These pure lines were kept in culture until June 6, when they had all died. Last fall, November 12, another stool specimen from the same case was procured and organisms from this have been kept in culture and are still flourishing (June, 1922).

History of the Case.—The patient from whom the material was obtained was a woman who had been suffering with chronic diarrhea for 13 years. All attempts to control it have failed. There is a triple infection of *Chilomastix mesnili*, *Trichomonas hominis*, and *Spirochæta eurygyrata*.[1]

[1] I am indebted to Dr. Charles Simon for the material and history of the case.

EXPERIMENTAL.

Frequency of Occurrence.—In this part of the country *Spirochæta eurygyrata* does not seem to be of frequent occurrence. A survey was made of 180 stools sent into the laboratories of the Johns Hopkins Hospital from 105 patients, but *Spirochæta eurygyrata* was not found in any of them.

In the eastern hemisphere the reports are quite different. Macfie (8) found all the persons examined in the Gold Coast colony to be infected, and notes that Carter, examining dysenteric soldiers, found 56.5 and 41.5 per cent of the non-dysenteric soldiers infected. Porter (10), who was also working in Africa, found twenty-nine spirochetal infections out of 93 cases examined and says they rank next to *Entamœba coli* infections in frequency of occurrence. Fantham (12) in a survey of 1,305 dysenteric patients, with a total number of 3,325 examinations, found spirochetes only 42 times; while Faust and Wassell (13) examined 359 cases in Wuchang and found 0.3 per cent infected with *Spirochæta eurygyrata*.

Culture Media.—The 1st year, Locke-egg, ovomucoid, and ox bile salts media were used. These had already proved excellent media for the cultivation of *Trichomonas hominis* (Hogue (14)) and *Waskia intestinalis* (Hogue (15)). The 2nd year, tubes containing 15 cc. of sterile 0.85 per cent sodium chloride solution and 0.3 cc. of sterile serum water were used. In the beginning, pig serum, diluted 1:4, and sheep serum, diluted 1:3, were both used, but it was soon found that the spirochetes lived longer and were more active in the medium containing pig serum.

The media used had a pH of 6.8, 7, and 7.4. In each of these the spirochetes grew, though the medium with a pH 7 gave the best results. The tubes were covered with layers of paraffin oil to prevent evaporation and to keep them from contact with the air.

Sterile pieces of rabbit kidney were put into some tubes of this sodium chloride-serum water medium and sealed with vaseline, but in these tubes the spirochetes did not multiply and lived only a short time. They are not anaerobic.

Age of Cultures.—In ovomucoid, Locke-egg, and ox bile salts media the spirochetes usually lived from 8 to 12 days. Transfers from these media were made every 2 or 3 days. In one rare case active spirochetes were found in ox bile salts medium 70 days after inoculation.

In the deep cultures of sodium chloride-serum water, covered with paraffin oil, they lived much longer. Here many cultures had active spirochetes after 46, 62, 64, and three after 94 days. One tube still contains many active spirochetes 127 days after inoculation. In this tube there are both long and short individuals, showing that division is still taking place and that the culture is in good condition. Evidently an equilibrium has been reached which completely satisfies the organisms.

Method. Isolation of Pure Lines.—Loopfuls of the semiliquid stool were put into test-tubes of various media and these were incubated at 35°C. In a few days there was a good growth of spirochetes of all sizes. From these cultures very small hanging drops were made and examined microscopically. When a drop was found which contained only one spirochete, the edges of the cover-slip were cut off and the remaining piece of glass, with its small drop of culture medium, was put into a test-tube with fresh medium. This spirochete was the beginning of a pure line; *i.e.*, a line derived from a single individual. This method was found better than isolation with the Barber pipette, since the spirochetes move very rapidly.

An attempt was made to free the spirochetes from the intestinal bacteria growing with them.

Nine large test-tubes containing 2 per cent agar, 0.7 per cent sodium chloride, and 0.4 per cent peptone were cooled to 50°C. To each tube 0.3 cc. of sheep or pig serum water was added. When hard, these tubes were inoculated with spirochetes by means of a capillary pipette and covered with paraffin oil to prevent drying. They were incubated at 35°C. 18 days later the test-tubes were broken into sterile Petri dishes and the agar cylinder was cut open. In the line of inoculation were many active bacteria and inactive spirochetes. Pieces of agar were cut from the sides of the column of growth and transplanted to tubes of sodium chloride-serum water. When these were later examined, no spirochetes were present. It is a question whether the supply of oxygen was insufficient for their maintenance, or whether they were unable to migrate into this dense medium.

Stains.—The spirochetes were stained with iron-hematoxylin, Giemsa's stain, Cross' stain (16), and weak carbolfuchsin. A loop of the culture containing a large number of the spirochetes was smeared on a cover-glass. This was killed in hot Schaudinn's fluid and then stained in iron-hematoxylin over night. With Giemsa's stain the

cover-slips were fixed in methyl alcohol. Cross' stain gave very good results and was a quick method. The smears on the slides were dried in the air and stained 2 minutes. Carbolfuchsin diluted also gave excellent results.

Vital Stains.—Neutral red, methylene blue, brilliant cresyl blue, Janus green, and pyrrol blue were all used. Varying concentrations of the stain were added to hanging drops of the culture. The cover-slips were sealed with vaseline and the spirochetes kept under observation often for 24 hours, but in no case were the organisms colored by these vital dyes.

Dark-Field Examination.—With dark-field illumination the spirochetes showed no flagella. Both ends were rounded. A dark line ran the entire length of the body in the midline. On either side of it were bright refractile zones. When the spirochetes were alive and active there were no cross-bars, or banding. As they moved a dark wave passed along the body from the anterior to the posterior end. This was only transitory.

In the old cultures in which the spirochetes were no longer active and therefore considered to be dead (Hogue (17)) they presented a very different aspect. The parallel lines of refraction were broken up. There were dark bars which went three-fourths or entirely across the body. The tops of the crests were usually very bright. It seemed as though the cytoplasm had become concentrated at these points.

These cross-bars, seen with dark-field illumination, of spirochetes in culture media, are further evidence for Noguchi's (18) theory "that the cross bands are formed from a homogeneous mass through sudden contraction." From his stained preparations he thinks it is "due to dehydration with absolute alcohol during fixation." In the present case the process is undoubtedly a slower one, taking place at the time of death. At this time the cytoplasm containing the diffuse chromatin material is concentrated at certain places along the body. When these are stained with iron-hematoxylin they take the chromatin stain, and have been interpreted by many as chromatin bars. In reality they are an artifact due to the coagulation of the protoplasm after death.

Effect of Different Media.—There was little morphological difference in the spirochetes grown in the different media. The very long forms

with over twenty crests were found in both pure lines (Strains S1 and S2), in Locke-egg and in ox bile salts media. There may have been some substance in these media which delayed or prevented division.

In the ox bile salts medium a larger number of the spirochetes seemed to be loosely coiled and flexible, and, consequently, to move rather with a snake-like than a corkscrew-like motion.

There was also a noticeable difference in the rate of growth in the media. The greatest number of individuals in a culture of ovomucoid appeared on the 2nd day, of Locke-egg on the 7th day, and of sodium chloride-serum water covered with paraffin oil on the 14th day or even later.

Motion.—The motion of the spirochete depends largely on its size and consequently on its age. The short and medium sized spirochetes are very active. There is the typical spirochete motion, the darting back and forth. As they grow longer this motion becomes impossible and one notices a wave-like, undulating motion which passes the length of the body and often appears as a twisting of the entire body from side to side. The body is not rigid but appears quite flexible, bending at any part. This wavy, almost worm-like, motion is always indicative of age. It becomes less and less until finally all motion ceases. If these organisms are transferred to fresh culture media there is no growth or multiplication, showing that they are dead. Here again motion and locomotion can be taken as a criterion of life, as I (17) have already shown to be the case for some other organisms.

Morphology.—Some former observers have quite rightly said that the length of the spirochete and the number and twist of the spirals cannot be taken as criteria for the determination of different species. The two organisms used to start the two pure lines (Strains S1 and S2) both had three crests. In the races derived from them were subsequently found individuals varying in length from 4 μ to 56 μ (Figs. 1 and 12). In the young cultures, about a week old, the spirochetes generally have one, two, or three crests (Figs. 1 to 6). These shorter forms increase in size until the very long forms (Figs. 12 and 14) appear in great numbers in cultures 3 or more weeks old. Some of these have as many as twenty-seven crests.

These older forms often have stretched spirals; *i.e.*, places where the coiling is irregular (Figs. 12 and 14). They frequently bend at these places (Fig. 14) while they are twisting from side to side.

There are also in these pure lines both Type I (Fig. 5) and Type II (Fig. 6) of Werner's spirochetes; *i.e.*, those which are loosely coiled and flexible, and those which are more tightly coiled and less flexible. However, I find Type I not so active as Type II, which with its tighter coils has more of the corkscrew motion of *Treponema pallidum*. Type I is also slightly thicker than Type II with its tighter coils.

In the literature there is a great discrepancy as to the kinds of ends which the spirochetes have. In all the spirochetes which were cultured in the present study both ends have been rounded. Occasionally a few of the short forms will have slightly pointed ends (Figs. 2, 3, and 5) but I have never observed sharply pointed ends, or ends drawn out into filaments.

The cross-bars appearing in stained preparations and described by so many observers of spirochetes have already been shown to be artifacts due to the coagulation of the protoplasm which contains diffuse chromatin. The dyes used were all basic and consequently stained the chromatin material. These bars were not very wide, nor were they of uniform width (Fig. 5). As a rule they were not arranged at regular intervals, though they were usually at the crests (Figs. 3, 5, and 6). In some cases the dark areas did not extend entirely across the spirochete (Fig. 4). This may be due to too much differentiation of the stain, but more probably the material was coagulated at one side of the organism, as appeared to be the case with dark-field illumination. One also notices in many of these stained preparations a massing of this deeply staining material at one or at both ends of the organism (Figs. 3 to 5 and 7). With weak carbolfuchsin and with Giemsa's stain the bars were visible, but were not nearly so distinct. With Cross' stain the whole organism was usually stained a deep purple, though, with shorter exposure to the stain, faint bars could be distinguished along the body.

Reproduction.—Transverse division is the only kind of reproduction which has been so far observed for *Spirochæta eurygyrata*. It may be equal or unequal. In the shorter forms it is usually equal (Figs. 7 and 9). In the longer forms it may be equal (Fig. 15), but is more

likely to be unequal (Figs. 8 and 11 to 13). Indeed it may be so unequal that it can almost be called fragmentation(Fig. 12). Some individuals were found which, after being stained with Cross' stain, showed several clear spaces (Fig. 16). These forms would probably fragment into several small individuals. This mode of reproduction, no doubt, accounts for the numerous short forms seen in the cultures a week old, which have been started by inoculating tubes from older cultures full of long forms.

Incurvation was occasionally seen (Fig. 10) and was interpreted as transverse division, the spirochete being twisted upon itself in its effort to separate into two individuals.

Porter (10) has described coccoid bodies which are formed and serve as a means of transmitting the organism. In the older cultures there are certain individuals which seem to contain one or more refractile granules, situated usually in the crests. With dark-field illumination these bodies were interpreted as being the concentration of the protoplasm due to death. They were never seen in active spirochetes in the dark field. Moreover, they have never been seen as separate bodies in the culture medium, nor have they been found with the young spirochete growing from them as has been so often described for other spirochetes.

Animal Experimentation.—It was hoped that rats could be used as hosts for these spirochetes, but after making daily fecal examinations of four rats for 8 days, it was found that two of them were already infected with a spirochete which appeared at irregular intervals in the stools. Other rats were examined and found infected, so it was decided not to use this animal.

Several cats were found infected with the spirochetes described by Kasai and Kobayashi (19), but finally by daily fecal examinations for 8 days three were found which were not infected. These were each fed 6 cc. of a culture of active *Spirochæta eurygyrata*. On 8 succeeding days their stools were examined microscopically and culturally but no spirochetes were found. 43 days later the cats were again examined but still with negative results. It seems evident that the cat cannot become infected through the mouth with this species of spirochete.

SUMMARY.

1. Pure lines of *Spirochæta eurygyrata* were isolated and cultured.

2. They lived longest in a medium which was made up of sodium chloride and pig serum water.

3. They stained readily with iron-hematoxylin, Giemsa's stain, Cross' stain, and carbolfuchsin.

4. They did not stain with the vital dyes used.

5. They divide by transverse division.

6. Transverse bars, which are seen in stained spirochetes and in dead ones with dark-field illumination, are considered coagulated protoplasm, due to death.

7. Coccoid bodies were not seen in living individuals nor were they seen free in the culture medium.

8. Cats were fed cultures of *Spirochæta eurygyrata* but did not become infected with them.

9. *Spirochæta eurygyrata* is not of frequent occurrence in this part of the United States.

BIBLIOGRAPHY.

1. Kowalski, H., *Centr. Bakt., Ite Abt., Orig.*, 1894, xvi, 321.
2. Le Dantec, *Compt. rend. Soc. biol.*, 1903, lv, 617.
3. Mühlens, P., *Z. Hyg. u. Infectionskrankh.*, 1907, lvii, 405.
4. Werner, H., *Centr. Bakt., Ite Abt., Orig.*, 1909, lii, 241.
5. Thomson, J. G., and Thomson, D., *Proc. Roy. Soc. Med.*, 1913–14, vii, Marcus Beck Lab. Rep., 47.
6. Fantham, H. B., *Brit. Med. J.*, 1916, i, 815.
7. Thomson, D., and Thomson, J. G., *J. Roy. Army Med. Corps*, 1916, xxvii, 1.
8. Macfie, J. W. S., *Lancet*, 1917, i, 336.
9. Luger, A., *Wien. klin. Woch.*, 1917, xxx, 1643.
10. Porter, A., A survey of the intestinal entozoa, both protozoal and helminthic, observed among natives in Johannesburg, from June to November, 1917, Memoir of the South African Institute for Medical Research, No. 11, Johannesburg, 1918.
11. Langendörfer, J. W., and Peters, *Münch. med. Woch.*, 1921, lxviii, 12.
12. Fantham, H. B., *Lancet*, 1916, i, 1165.
13. Faust, E. C., and Wassell, C. M., *China Med. J.*, 1921, xxxv, 532.
14. Hogue, M. J., *Am. J. Trop. Med.*, 1921, i, 211.
15. Hogue, M. J., *J. Am. Med. Assn.*, 1921, lxxvii, 112.
16. Cross, H. B., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 51.

17. Hogue, M. J., *J. Exp. Zool.*, 1922, xxxv, 1.
18. Noguchi, H., *J. Exp. Med.*, 1921, xxxiv, 295.
19. Kasai, K., and Kobayashi, R., *J. Parasitol.*, 1919–20, vi, 1.

EXPLANATION OF PLATE 57.

All the drawings were made with a camera lucida. Magnification, × 3,000.

FIG. 1. A short *Spirochæta eurygyrata* stained with carbolfuchsin.

FIG. 2. A short form with pointed ends, stained with Cross' stain.

FIG. 3. A short form stained with iron-hematoxylin to show the cross-bars.

FIG. 4. A short form stained with iron-hematoxylin. The bars do not extend entirely across the body.

FIGS. 5 and 6. Short forms showing the cross-bars extending across the body. Stained with iron-hematoxylin.

FIG. 7. A short form preparing for equal transverse division. There is a massing of the deeply staining material at each end. Stained with iron-hematoxylin.

FIG. 8. A longer form dividing by unequal transverse division. Cross' stain.

FIG. 9. A short form dividing by equal transverse division. Stained with carbolfuchsin.

FIG. 10. Incurvation, interpreted as transverse division. Cross' stain.

FIG. 11. A form dividing by unequal transverse division. Stained with carbolfuchsin.

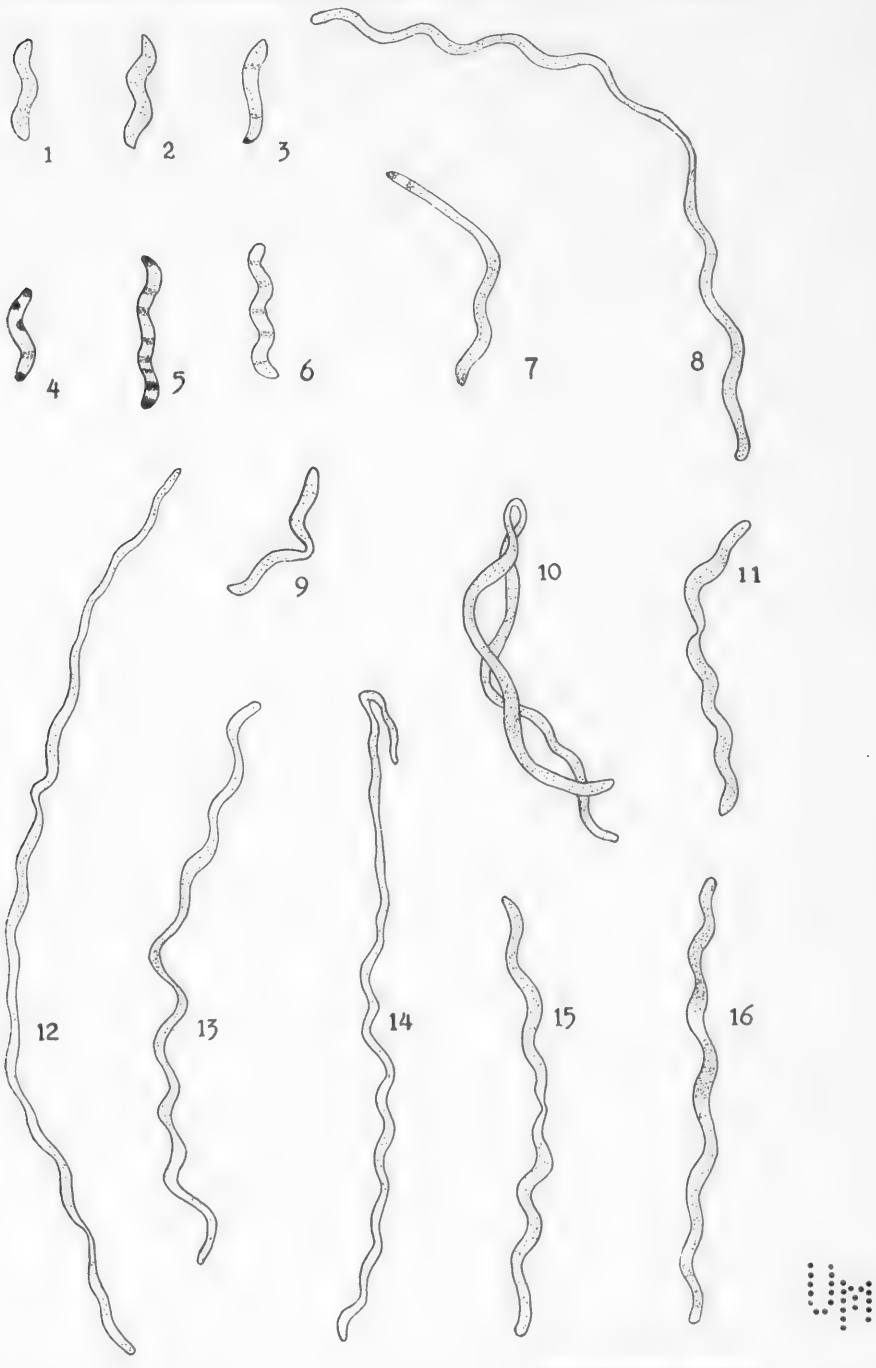
FIG. 12. A long form dividing by very unequal division. Cross' stain.

FIG. 13. A long form dividing by slightly unequal division. Cross' stain.

FIG. 14. The bending of the spirochete at the place where unequal division is taking place. Iron-hematoxylin stain.

FIG. 15. A spirochete dividing by equal transverse division. Cross' stain.

FIG. 16. A spirochete preparing to divide into six smaller individuals. Cross' stain.



(Hogue: *Spirochæta eurygyrata.*)

INSUSCEPTIBILITY TO SENSITIZATION AND ANAPHYLACTIC SHOCK.

By WARFIELD T. LONGCOPE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 10, 1922.)

The object of the experiments that are to be described was to gain some information concerning the factors which determine the variations that species and individuals show towards certain forms of intoxication. The study forms a continuation of some investigations previously made with Mackenzie (1) and later completed by Mackenzie and Leake (2). In these previous investigations the immunological processes involved in the production of serum disease in the human being were selected as the problem.

It is well known that the incidence of serum disease following the injection of therapeutic sera increases roughly in proportion to the amount of serum injected, so that up to a certain point the susceptibility to serum disease is dependent upon the size of the dose. After a dose of approximately 75 to 100 cc. of serum is reached the incidence of serum disease remains about stationary at 80 to 90 per cent, and no matter how much more serum is administered, a small proportion of individuals always escapes the manifestations of serum sickness.

Recently, Coca, Deibert, and Menger (3) have shown, moreover, that the North American Indian is relatively much less susceptible than the white race, for they state that of twenty-six healthy Indians each of whom received 100 cc. of normal horse serum intravenously, only twelve, or 46 per cent, developed symptoms of serum sickness, and in most of these instances the disease ran a short and very mild course, averaging only 2 days.

It was desired in the previous experiments to study the protective mechanism possessed by the small group of white patients that escaped serum sickness. These individuals were found to differ from the susceptible inasmuch as they did not produce demonstrable antibodies in the form of precipitins and anaphylactic antibody, and inas-

much as the antigen, or horse serum, remained in the circulation as an apparently innocuous substance for periods of weeks or months after it was injected. In the susceptible individuals precipitins were usually formed in large amounts, and the antigen disappeared rapidly from the circulation with the subsidence of serum disease. It seemed, therefore, that the insusceptible individual possessed some mechanism which inhibited the union of antigen with the cells of the body and thus prevented the effects which follow the rapid and usual reactions between the foreign protein and the tissues of the host. According to Metchnikoff, a similar form of mechanism explains the insusceptibility of certain cold blooded animals to diphtheria and tetanus toxin. And according to Coca, Russell, and Baughman (4) the partial impermeability of the cells of the rat to diphtheria toxin explains their great resistance to this poison.

Individual variation to sensitization by foreign proteins is known to occur in all laboratory animals but in the guinea pig or even the rabbit, it is so rare that experiments with these animals seemed unprofitable.

The white rat, on the other hand, has generally been considered as an animal which could not be made anaphylactic (Doerr (5)). It is not possible, however, to find many observations in the literature which afford much information on this subject.

Arthus (6) simply states that he has observed anaphylactic phenomena in white rats after repeated injections of foreign sera. Rosenau and Anderson (7) mentioned the fact that they had tested the anaphylactic reaction in monkeys, rabbits, mice, dogs, cats, rats, chickens, and pigeons and stated that they obtained positive results with the dog, rabbit, and cat. Uhlenhuth, Haendel, and Steffenhagen (8) reported that they were unable to produce anaphylactic shock in white rats or mice. These observations were confirmed by Trommsdorff (9), who failed in attempts to sensitize twenty-five white rats and twenty-five mice to egg white or to horse serum. The second injection of these proteins was administered intravenously after intervals of 5 days to 12 weeks. But the most complete study of this subject has probably been made by Novy and De Kruif (10), who, in attempting to sensitize white rats to horse serum, beef serum, rabbit serum, and egg white, found that the second injection made intravenously of such large amounts of the undiluted antigens as 2 to 4 cc. in these supposedly sensitized rats did not produce any symptoms which could definitely be considered as anaphylactic, and was never fatal. When, on the other hand, antigens diluted with distilled water were given intravenously in amounts of

7 to 10 cc. at the second injection, violent symptoms occurred and in many animals were followed by death. In the normal rat similar amounts of the mixture of antigen and distilled water produced little untoward effect. The intoxication brought about in the supposedly sensitized rat, by the second injection of diluted antigen, proved, however, to be non-specific, for it could be produced quite as readily by the injection of an equal amount of distilled water without antigen. Novy and De Kruif concluded, therefore, that though the rat was practically insusceptible to specific anaphylactic shock, the repeated injection of a foreign protein, nevertheless, produced some change which rendered the animal highly sensitive to the intravenous injection of distilled water and gave rise to a state which was conducive as well to a much more rapid formation of anaphylatoxin in the serum of the shed blood than could be obtained with normal rat serum. Novy and De Kruif (11) also studied the effect upon white rats of the intravenous injection of anaphylatoxin, agar sol-gel, and peptone. To all of these substances the rats were much more refractory than guinea pigs. Weight for weight, the rat tolerated 100 times as much anaphylatoxin as guinea pigs, three times as much agar sol-gel, and about seven times as much 10 per cent peptone solution. For white rats the lethal dose of 10 per cent solution of Witte's peptone was 2 gm. per kilo.

From these experiments it seems evident that the white rat is both highly refractory to anaphylaxis and very resistant to the action of such toxic substances as anaphylatoxin and peptone.

EXPERIMENTAL.

In the present investigation the following plan of procedure was adopted: (1) study of the symptoms produced in white rats by intravenous injection of peptone and histamine; (2) attempts to sensitize white rats to horse serum; (3) determination of the state of sensitization (*a*) by intravenous and subdural injection of antigen, (*b*) by skin reactions, and (*c*) by the uterine strip reaction; (4) attempts to sensitize passively guinea pigs with the serum of white rats immunized to horse serum; and (5) study of the antigen-antibody content of the serum of white rats injected with horse serum.

Symptoms Produced by the Intravenous Injection of Peptone and Histamine.

In order to obtain some criteria of shock similar to that in anaphylaxis, white rats weighing approximately 100 to 125 gm. were given intravenous injections of solutions of Witte's peptone and of histamine.

1 gm. of Witte's peptone was dissolved by heat in distilled water, centrifuged, and the supernatant fluid employed for injection. The histamine was made up in 1 and 2 per cent solution in 0.85 per cent NaCl.

The intravenous injection of 0.1 gm. of peptone per 100 gm. of body weight caused very severe symptoms. Immediately after the injection the animals went into collapse. They lay flat upon the table with all four legs spread out; the respirations were slow and difficult. After 2 to 3 minutes they often made an attempt to stand but failed; within 4 or 5 minutes there were sometimes slight convulsive movements with irregular gasping respirations; in about 10 minutes the respirations would become rapid (68 in one animal), while the paralysis and spasmodic convulsive movements continued. One animal recovered after this dose at the end of 2 hours. A second animal died in 10 minutes.

The effects following the intravenous injection of histamine were quite similar; immediate collapse, gasping respiration, paralysis, convulsive seizures, fall in temperature, the expulsion of feces and urine, and the discharge of fluid from the nose were all prominent features. One noteworthy symptom was an extreme and acute exophthalmos. In those animals that recovered, the severe symptoms persisted for an hour or longer. Two rats withstood a dose of 10 mg. of histamine per 100 gm. of body weight, two animals 15 mg. of histamine per 100 gm. of body weight, and one animal died after receiving 30 mg. of histamine per 100 gm. of body weight.

Attempts to Sensitize to Horse Serum.

A criterion having been established for symptoms which by analogy with other animals should simulate anaphylaxis in the white rat, attempts were made to sensitize white rats by repeated injections of horse serum given subcutaneously, intraperitoneally, intravenously, or subdurally. Twenty-seven experiments in twenty-three rats weighing from 75 to 150 gm. were devised for this purpose. Thirteen rats were given three injections of from 0.1 to 0.5 cc. of horse serum subcutaneously or intraperitoneally at 2 to 3 day intervals, three rats six injections of similar amounts, two rats a single injection of 0.5 cc. intravenously, and five rats three injections subcutaneously or intra-

TABLE I.

Rat No.	Weight.	Date sensitized.	Amounts of horse serum.	Method.	Length of time after last injection.	Amount of horse serum.	Method.	Symptoms.
	gm.	*1921*	*cc.*		*days*	*cc.*		
1	85	Oct. 5	0.5	Subcutaneous.	21	2.5	Intraperitoneal.	None.
		" 7	0.5	"				
		" 10	0.5	"				
2	84	" 5	0.5	"	22	0.5	Intravenous.	"
		" 7	0.5	"				
		" 10	0.5	"				
4	94	" 6	0.5	Intraperitoneal.	21	0.5	"	"
		" 7	0.5	"				
		" 10	0.5	"				
6	83	" 5	0.5	"	22	0.5	"	"
		" 7	0.5	"				
		" 10	0.5	"	(Jan. 5, 1922.) 66	1.0	"	"
7	154	" 17	0.1	Subcutaneous.	29	0.6	"	"
		" 20	0.25	"				
		" 24	0.25	"				
8	138	" 17	0.1	"	29	0.5	"	No definite symptoms. Remains quiet; respirations a trifle irregular.
		" 20	0.25	"				
		" 24	0.25	"				

TABLE I—*Continued.*

Rat No.	Weight.	Date sensitized.	Amounts of horse serum.	Method.	Length of time after last injection.	Amount of horse serum.	Method.	Symptoms.
	gm.	*1921*	*cc.*		*days*	*cc.*		
11	136.5	Oct. 17	0.1	Intraperitoneal.	29	0.5	Intravenous.	None.
		" 20	0.25	"				
		" 24	0.25	"				
13	77	" 24	0.2	"	31	1.0	"	"
		" 26	0.4	"				
		" 28	0.5	"				
14	68	" 24	0.2	Subcutaneous.	31	0.5	"	Lies flat 5 to 10 min. after injection.
		" 26	0.4	"				
		" 28	0.5	"				
15	106	" 24	0.2	"	31	0.8	"	Lies flat 5 min. after injection. Seems weak. 15 min., recovery.
		" 26	0.4	"				
		" 28	0.5	"				
15	150	Nov. 29	0.8	Intravenous.	38	1.0	"	None.
16	83	Oct. 24	0.25	Intraperitoneal.	31	1.0	"	"
		" 26	0.5	"				
		" 28	0.5	"				
17	93	" 24	0.2	"	31	1.0	"	"
		" 26	0.5	"				
		" 28	0.5	"				

18	89	Oct. 24	0.2	Intraperitoneal.	31	1.0	Intravenous.	None
		" 26	0.5	"				
		" 28	0.5	"				
19	98	Nov. 2	0.5	Intravenous.	21	0.5	"	"
					44	0.5	Intraperitoneal.	"
20	150	" 2	0.5	"	21	0.5	Intravenous.	"
					44	0.5	Intraperitoneal.	"
		1922						
33	92	Jan. 5	0.2	"	27	1.0	Intravenous.	"
		" 9	0.5	Intraperitoneal.				
		" 11	0.5	"				
		" 13	0.5	"				
		" 16	0.5	"				
		" 18	0.5	"				
41	125	" 6	0.2	"	27	1.0	"	Seems slightly weak but walks well. No respiratory difficulty.
		" 9	0.5	"				
		" 11	0.5	"				
		" 13	0.5	"				
		" 16	0.5	"				
		" 18	0.5	"				
Controls.					*1921*			
21	127				Nov. 28	1.0	"	None.
22	78				" 28	1.0	"	"
23	140				" 28	1.0	"	"
19	98				" 2	0.5	"	"
20	150				" 2	0.5	"	"

TABLE II.

Rat No.	Weight.	Date sensitized.	Amounts of horse serum.	Method.	Length of time after last injection.	Amount of horse serum.	Method.	Symptoms.
	gm.	*1921*	*cc.*		*days*	*cc.*		
13	74	Oct. 24	0.2	Subcutaneous.	38	0.2	Subdural.	General convulsion; rapid respirations. 4 min., weak. 10 min., still collapsed; weak on left side. 15 min., recovered but paralyzed on left side.
		" 26	0.4	"				
		" 28	0.5	"				
		Nov. 28	1.0	Intravenous.				
14	68	Oct. 24	0.2	Subcutaneous.	38	0.2	"	None.
		" 26	0.4	"				
		" 28	0.5	"				
		Nov. 28	0.5	Intravenous.				
16	75	Oct. 24	0.2	Intraperitoneal.	38	0.5	"	Convulsions; stops breathing. 2 min., recovered. 3 min., gasping. 10 min., dyspnea continued. 1 hr., recovered.
		" 26	0.5	"				
		" 28	0.5	"				
		Nov. 28	1.0	Intravenous.				
17	100	Oct. 24	0.2	Intraperitoneal.	38	0.5	"	Moderate collapse; stops breathing. Respiration started in 1 min. 3 min., crawls; respirations rapid. 6 min., walks about recovering.
		" 26	0.5	"				
		" 28	0.5	"				
		Nov. 28	1.0	Intravenous.				
18	105	Oct. 24	0.2	Intraperitoneal.	38	0.3	"	Immediate collapse. Respiration starts in 1 min. 2 min., deep breathing. 3 min., labored respirations. 7 min., stands; respirations difficult. 18 min., recovered.
		" 26	0.5	"				
		" 28	0.5	"				
		Nov. 28	1.0	Intravenous.				

		1922						
42	120	Jan. 6	0.2	Intraperitoneal.	27	0.2	Subdural.	Walks about; respirations rapid and slightly difficult. Recovered in 3 min.
		" 9	0.5	"				
		" 11	0.5	"				
		" 13	0.5	"				
		" 16	0.5	"				
		" 18	0.5	"				
Controls.								
32	105					0.5	"	Immediate collapse; convulsions. 2 min., lies on side; hind leg weak. 7 min., better. 15 min., recovery.
33	92					0.2	"	Immediate convulsions; respiration stopped. 2 min., rapid respirations; recovery. 10 min., recovered.
34	105					0.5	"	Immediate shivering; respiration ceases. 2 min., breathing. 3 min., respirations difficult. 6 min., still very sick. 7 min., recovery.
60	115					0.2	"	Immediate collapse; respirations rapid; sneezing; coughing. 6 min., respirations difficult; fluid from nose. 10 min., sneezing; respirations difficult. 30 min., still very sick. 1 hr., recovery.

peritoneally and 1 month later 1 cc. intravenously. The test injections were made from 21 to 44 days after the last sensitizing dose. These experiments are recorded in Tables I and II. In seventeen experiments the injection of 0.5 to 1 cc. of horse serum into the femoral vein of these supposedly sensitized rats (Table I) seemed quite harmless and never produced any symptoms that resembled in the least those obtained with peptone or histamine, much less death. When injections of from 0.2 to 0.5 cc. of horse serum were made subdurally in six rats, immediate and violent symptoms usually occurred, which, however, never proved fatal, and exactly similar symptoms were obtained in the normal control rats (Table II).

From these experiments one must conclude that anaphylaxis to horse serum cannot be obtained in white rats by a second injection of antigen given intravenously or subdurally.

Determination of the State of Sensitization.

The skin of seventeen white rats subjected to repeated injections of horse serum given subcutaneously, intraperitoneally, or intravenously was tested for a specific reaction on the 20th to the 63rd day after the last injection of antigen. The sides of the rat were shaved 2 or 3 days preceding the experiment and an injection of 0.02 cc. of horse serum diluted 1:10 with 0.85 per cent NaCl was made intracutaneously. As controls, similar amounts of rabbit serum diluted 1:10, or of 0.85 per cent NaCl, were given intracutaneously to the inoculated rats and at the same time both horse serum and rabbit serum in similar amounts and dilutions were injected intracutaneously into normal rats. The skin of the rats was observed every 15 minutes for a period of 1 to 2 hours and again at the end of 12, 24, and after 48 hours. In no instance was there any evidence of a skin reaction such as may be obtained in the guinea pig, rabbit, or in man, and in no instance did the test injection produce an effect which differed from that in the controls.

It must be concluded, therefore, that the skin of the white rat subjected to repeated injections of horse serum does not differ from that of the normal rat in its reaction to subsequent intracutaneous injections of horse serum in the amounts utilized.

It is generally assumed that the reactions of the smooth muscle of the sensitized virgin guinea pig as employed by Dale is one of the most delicate tests for sensitization; and for this reason six virgin white rats averaging 50 to 60 gm. were injected intraperitoneally with 0.2, 0.2, 0.5, and 0.5 cc. of horse serum every other day, and after intervals of from 38 to 54 days the uteri were removed, suspended in 250 cc. of oxygenated Locke's solution at 39°C., and tested for their reaction to horse serum. As control antigens, rat serum, rabbit serum, and sheep serum were employed. In five control experiments with the uteri of normal virgin rats, it was found that horse serum in doses of from 1 to 2.5 cc. produced no change in the regular rhythmic uterine contraction but sometimes resulted in a very slight general shortening of the muscle strip which was made evident by an elevation of the line of contractions upon the surface of the drum. The effect of histamine in doses of 1 mg. was to produce immediate relaxation of the muscle strip with great slowing of the contraction, which often became irregular.

The effect of horse serum on the uteri of the inoculated rats in doses of 1 cc. did not differ from that produced by horse serum upon the uteri of normal rats. No unusual contractions, such as are seen in the guinea pig uterus, were observed, and in no instance was the relaxing effect noted with histamine encountered.

It was therefore concluded that the uteri of white rats receiving repeated injections of horse serum did not differ in their reaction to horse serum from those of normal rats.

It seemed possible to conclude, therefore, from these series of experiments that white rats could not be made anaphylactic to horse serum, as tested by several methods. The next step was to determine upon what this refractory condition depended.

This insusceptibility might be explained by at least one of two hypotheses: first, that the tissues of the rat were insusceptible to the poisonous effect which follows the reaction presumably of antibody and antigen in the highly sensitized animal when a second injection of antigen is administered; or, second, that the tissues of the rat were for some reason unable to form an hypothetical anaphylactic antibody or were in some way prevented from accomplishing this.

To answer the first question, experiments were done to determine whether the serum of supposedly immunized rats contained anaphylactin and was capable of transferring passive anaphylaxis to guinea pigs.

Attempts to Sensitize Passively Guinea Pigs with the Serum of White Rats Immunized to Horse Serum.

The serum of fourteen white rats, subjected to repeated intraperitoneal injections of horse serum, was injected within a few hours of bleeding into guinea pigs. Seven guinea pigs received from 0.4 to 0.6 cc. of rat serum intravenously and seven from 0.75 to 1 cc. intraperitoneally. After an interval of 23 to 25 hours these guinea pigs were given 0.5 cc. of horse serum intravenously. No symptoms which could be interpreted as those of anaphylactic shock were observed in any instance. The experiments are summarized in Table III.

These experiments furnish a strong argument, in spite of the resistance of the white rat to peptone and histamine, against the idea that the rat behaves immunologically in the same manner as the guinea pig, rabbit, and dog, but nevertheless escapes the symptoms of anaphylaxis owing to its resistance to the poisonous effects of the shock. It was, consequently, necessary to seek further for an explanation of its insusceptibility to anaphylaxis.

Antigen-Antibody Content of the Serum of White Rats Injected with Horse Serum.

Experiments were therefore instituted to determine whether the second hypothesis was correct and to discover if the same protective mechanism towards sensitization existed in the rat as has been described for the human being. For this purpose a study of the antigen content of the serum of rats injected with horse serum was made. Twenty-one rats were used. Since the size of the rat prohibited the use of a single rat for repeated bleedings, it was necessary to use a single rat for each experiment. The rats, therefore, were killed by bleeding from the heart at various times after a single intravenous injection of approximately 1 cc. of horse serum per 100 gm. of body weight. By employing the precipitin reaction in the manner described

TABLE III.

Guinea pig No.	Weight.	Date.	Rat serum No.	Amount of rat serum.	Method.	Interval.	Amount of horse serum injected intravenously.	Symptoms.
	gm.	*1922*		*cc.*		*hrs.*	*cc.*	
6	240	Jan. 13	8	1.0	Intraperitoneal.	23	0.5	None.
5	200	" 13	15	0.6	Intravenous.	23	0.5	Shivering; no other symptoms.
10	230	" 19	19	0.5	"	24	0.5	None.
11	240	" 19	20	0.5	"	24	0.5	"
12	180	" 20	35	0.5	"	23	0.5	"
13	190	" 20	36	0.5	"	23	0.5	Shivering; no other symptoms.
14	200	Feb. 1	37	0.4	"	23	0.5	None.
15	225	" 1	38	0.5	"	23	0.5	"
		1919						
19	150	Mar. 8	33	0.75	Intraperitoneal.	24	0.5	Shivering; slight weakness of hind legs. Recovered in 3 min.
20	111½	" 8	34	1.0	"	24	0.5	None.
21	146	" 8	41	1.0	"	24	0.5	"
22	136	" 8	42	1.0	"	24	0.5	Slight shivering; slight weakness; immediate recovery.
23	120	" 8	59	0.2	"	25	0.5	Shivering.
24	223	" 8	60	1.0	"	25	0.5	None.
Controls.								
8	250	Jan. 13					0.5	"
7	230	" 13	Normal.	0.8	Intraperitoneal.	23	0.5	"
9	250	" 20	"	0.5	Intravenous.	48	0.5	"
17	190	Feb. 1	"	0.5	"	23	0.5	Shivering.
25	120	Mar. 8	"	1.0	Intraperitoneal.	24	0.5	None.
						days		
1	220	Jan. 19	Jan. 6. Sensitized to horse serum.			13	0.5	Immediate symptoms; death in 2 min.
2	230	Feb. 1	" 6. " " " "			25	0.5	" " " " 3 "
3	220	Mar. 8	" 6. " " " "			61	0.5	Death in 2 min.

in previous communications—except that all sera were employed in amounts of 0.25 cc.—it could be shown that antigen (horse serum) when injected intravenously did not persist in the circulation but disappeared in from 12 to 14 days, a period which is approximately that required by the rabbit to dispense with a similar antigen.

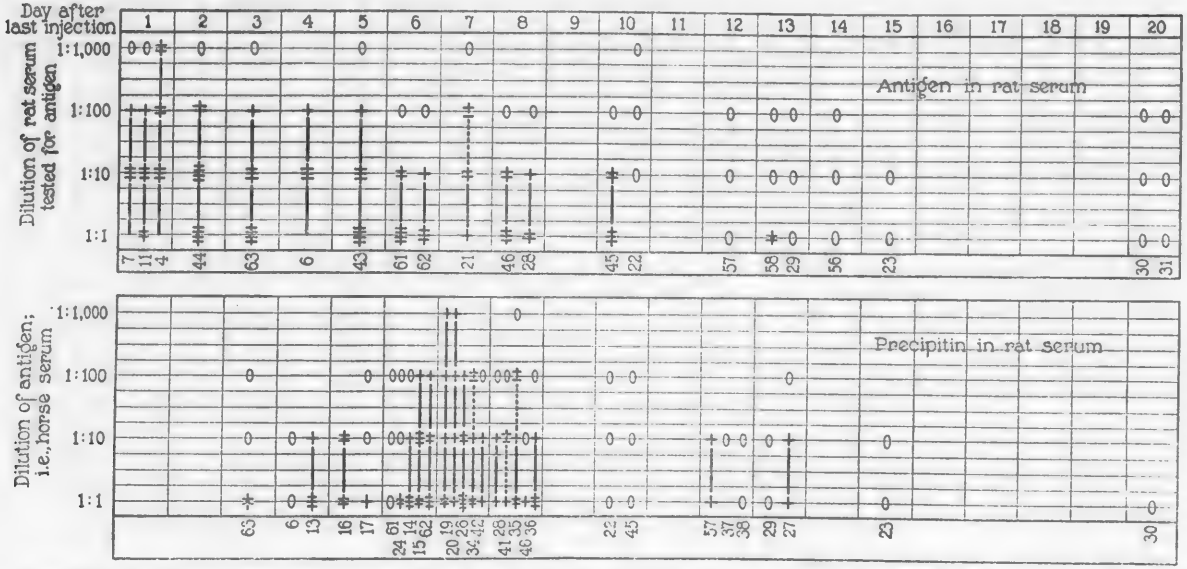
It was thought that the kidney of the rat might be permeable to the horse serum and thus allow of its escape from the body, but precipitin reactions upon the urine of white rats injected with horse serum failed to demonstrate antigen in the urine from the time of injection to the time of the disappearance of the horse serum from the circulation.

It was necessary, therefore, to search for some other explanation. Although it seemed *a priori* improbable that the white rat which was refractory to sensitization would form precipitins, still tests were made for specific precipitins in the serum of twenty-eight white rats receiving single and repeated injections of horse serum, and it was found that these animals were capable of forming such precipitins in fairly high concentration. Precipitins for horse serum could be demonstrated in the serum of twenty of the twenty-eight rats, appearing first on the 3rd to the 4th day after the last of a series of inoculations and increasing in intensity up to the 6th or 8th day, when they were present with great regularity. After this time, there was a rapid decline in positive reactions, for the sera from only two of nine rats drawn after the 10th day showed precipitins. It was also found that it was during the high concentration of precipitins in the serum that the antigen disappeared from the circulation. A graphic presentation of the precipitinogen-precipitin balance is shown in Text-fig. 1.

The mechanism of precipitin formation and the relationship between precipitin and precipitinogen (horse serum) proved, therefore, to be much the same as it is in the rabbit.

Thus, though sensitization to horse serum did not occur, the cells of the white rat had apparently an avidity for horse serum which was as great as, or even greater than that of the rabbit.

Though there has been considerable discussion as to the identity of the anaphylactic antibody and precipitin, the work of Doerr and Russ (12) in rabbits and the subsequent observations of Weil (13) have lent such strong support to the idea that these two bodies are one and the same, that most investigators (Doerr (5), Zinsser (14), Wells (15))



TEXT-FIG. 1. Graphic representation of the precipitinogen-precipitin balance. Each upward stroke represents the test upon the serum of a single rat. The cross lines on these strokes indicate the intensity of the reaction at the given dilution.

have accepted this explanation as the correct one. Doerr in particular, after a complete review of the literature, has come to this conclusion. It has, however, been generally known that the ease with which an animal may be sensitized to foreign proteins and the readiness with which it forms specific precipitins are not always parallel. The guinea pig, for instance, is highly subject to sensitization but is an extremely poor precipitin producer, whereas the rabbit, which is much more difficult to sensitize, produces precipitins in large amounts.

The present experiments seem to demonstrate that in white rats precipitins and anaphylactin are not identical and that good precipitin formation can occur without anaphylactic sensitization and without the appearance of anaphylactic antibody in the serum of the rat.

The differences which several species of animals show in the ease with which they may be sensitized to foreign proteins and the readiness with which they may be subjected to shock by a second injection of the same antigen have already been alluded to, and it now seems from these experiments that the rat stands at one end of the scale, as a completely refractory animal, and probably the guinea pig at the other, as representing the most highly susceptible animal. This refractory condition of the rat does not seem to be due to a resistance of specialized tissues to the toxic effect of anaphylactic shock, or to an inability of the tissues to absorb antigen, for on the one hand, the animal may be killed by histamine or peptone in large doses, and on the other, the tissues are capable of producing precipitins in considerable concentration but not anaphylactin. The explanation seems rather to be found in the fact that the preliminary injections of antigen, though capable of calling forth precipitins, fail to prepare the cells of the body in the manner necessary to make them vulnerable either by an antibody-antigen reaction or by some other mechanism to the second injection of antigen.

SUMMARY.

1. Attempts to produce anaphylactic shock in white rats by second intravenous or subdural injections of horse serum have failed.

2. It was impossible to demonstrate either by skin reactions or by the uterine reaction that white rats can be sensitized to horse serum.

3. It was not possible to sensitize guinea pigs passively with the serum of white rats presumably immunized to horse serum.

4. In spite of the fact that the white rat could not be made anaphylactic to horse serum, the tissues of the animal reacted with the horse serum to form precipitins in fair concentration and the antigen disappeared from the circulation soon after the precipitins reached their greatest concentration in the blood.

5. These experiments would indicate that in the white rat anaphylaxis and precipitin formation are independent and represent different types of immunological processes.

BIBLIOGRAPHY.

1. Longcope, W. T., and Mackenzie, G. M., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 133.
2. Mackenzie, G. M., and Leake, W., *J. Exp. Med.*, 1921, xxxiii, 601.
3. Coca, A. F., Deibert, O., and Menger, E. F., *J. Immunol.*, 1922, vii, 201.
4. Coca, A. F., Russell, E. F., and Baughman, W. H., *J. Immunol.*, 1921, vi, 387.
5. Doerr, R., *Ergebn. Hyg., Bakt., Immunitätsforsch. u. exp. Therap.*, 1922, v, 71.
6. Arthus, M., *Compt. rend. Soc. biol.*, 1903, lv, 819.
7. Rosenau, M. J., and Anderson J. F., *Bull. Hyg. Lab., U. S. P. H., No. 29*, 1906, 57.
8. Uhlenhuth, Haendel, and Steffenhagen, *Centr. Bakt., Ite Abt., Ref.*, 1910, xlvii, suppl., 158.
9. Trommsdorff, R., *Arb. k. Gsndhtsamte*, 1909, xxxii, 560.
10. Novy, F. G., and De Kruif, P. H., *J. Infect. Dis.*, 1917, xx, 776.
11. Novy, F. G., and De Kruif, P. H., *J. Infect. Dis.*, 1917, xx, 618, 629. Novy, F. G., De Kruif, P. H., and Novy, F. O., *J. Infect. Dis.*, 1917, xx, 657.
12. Doerr, R., and Russ, V. K., *Z. Immunitätsforsch., Orig.*, 1909, iii, 181, 706.
13. Weil, R., *J. Immunol.*, 1916, i, 19, 35, 47.
14. Zinsser, H., Infection and resistance, New York, 1918.
15. Wells, H. G., *Physiol. Rev.*, 1921, i, 44.

LEUCOCYTIC SECRETIONS.

By ALEXIS CARREL, M.D., and ALBERT H. EBELING, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 14, 1922.)

I.

INTRODUCTION.

Leucocytes may be regarded as mobile unicellular glandular bodies which set free their secretions in the humors of the organism. But little is known of the nature and functions of the substances they secrete. In the following experiments, leucocytes were cultivated *in vitro* and a study was made of certain modifications of the medium caused by their secretions.

II.

Technique.

The leucocytes were obtained from hen blood and cultivated in Gabritschewski dishes. The serum was extracted from the culture medium and the variations of its inhibiting action on homologous fibroblasts, and of its lytic action on foreign erythrocytes, were measured.

1. Preparation of the Leucocytes.—The blood was taken from young adult chickens which had fasted for 24 hours. The animals were bled from the carotid artery or from one of the veins of the wing, through an oiled cannula or syringe. The blood was received in cold paraffin-coated tubes and centrifuged in ice for 10 minutes. After the plasma had been removed, a few drops of Ringer solution containing a small amount of embryo juice were placed at the surface of the corpuscles and the tubes were allowed to stand at room temperature for about 15 minutes. Then the film of leucocytes, which covered the erythrocytes, became solid, and could be removed to a watch-glass filled with Ringer solution. The film was washed almost free of erythrocytes and cut in small fragments of about equal size.

2. Preparation of the Medium.—The medium consisted of chicken plasma and embryo tissue juice. Its composition was generally as follows:

Chicken plasma	5.00 volumes.
Embryonic tissue juice	0.25 volume.
Tyrode solution	2.75 volumes.
Distilled water	2.00 volumes.

The medium was rendered hypotonic on account of the evaporation which takes place in the Gabritschewski dishes. In some cultures, casein was added to the medium. 1 gm. of casein was dissolved in a diluted solution of sodium hydroxide, and dialyzed against tap water for 3 days. Then it was again brought into solution by the addition of a little alkali, and rendered isotonic by a proper amount of NaCl. The pH of the solution was about 7.8. The amount of the solution used in the medium varied from 0.1 to 1 volume of a 1 per cent casein solution; that is, the concentration of casein was from 0.1 to 1 per 1,000.

3. Preparation of the Cultures.—The cultures were made on Gabritschewski dishes, the covers of which had been replaced by mica plates. The fragments of leucocytic films were suspended in 5 volumes of Tyrode solution, distilled water, and embryo juice in the concentrations previously described. The same number of fragments was used for each culture. Then 5 volumes of plasma were spread on the mica plate, with the necessary precautions to prevent bacterial contamination from the air. Afterwards, the leucocytic suspension was dropped from a pipette into the plasma, and thoroughly mixed with it. Care was taken to distribute the fragments of leucocyte film evenly. Their number was about thirty to each dish, in order that the colonies might grow freely around each fragment, without being in contact with the edges of the neighboring colonies before 48 hours. Controls were prepared consisting of the medium without leucocytes. Both cultures and their controls were incubated for 48 hours at 38°C. Other cultures and controls were placed in the refrigerator for the same time.

4. Preparation of the Serum from the Cultures.—After 48 hours, the cultures were examined. Around each fragment, a large colony of leucocytes had grown. The edges of the colonies were generally in close contact. Microscopic examination showed whether the cells

were living and in active condition, and whether bacterial contamination had occurred. Then the boxes were opened and the coagulum was sliced with fine scissors. The fluid and the fragments of coagulum were centrifuged at high speed for 10 minutes. The supernatant fluid was removed and its H ion concentration ascertained by the technique described by Felton.[1] The serum of the cultures of leucocytes always became slightly acid after 48 hours in the refrigerator. When the colonies had grown actively in the incubator for 48 hours, the acidity of the medium was much more marked. The pH of the serum from the cultures was brought to about that of normal serum by adding a small amount of sodium hydroxide.

5. *Action of Serum on Chicken Fibroblasts and on Foreign Erythrocytes.*—The sera from cultures with and without leucocytes, incubated or kept in the refrigerator, were compared from the standpoint of both their hemolytic effect on sheep or rabbit erythrocytes and their inhibiting power on chicken fibroblasts. The hemolytic action is expressed in the tables by the amount of serum required for a given degree of hemolysis. The action on chicken fibroblasts was ascertained by the rate of growth of a 10 year old strain of fibroblasts in a medium composed of 2.5 volumes of chicken plasma, 5 volumes of serum from leucocyte cultures, 2.2 volumes of Tyrode solution, and 0.3 volume of embryonic juice. In the tables, the rate of growth is indicated by the relative increase of the fragment of fibroblasts in 48 hours. The quotient of the average rate of growth of the experiment by the average rate of growth of the control expresses the action of serum on homologous fibroblasts.

III.

RESULTS.

The experiments consisted in measuring the inhibiting effect on chicken fibroblasts and the hemolytic power on sheep erythrocytes of the serum from media without leucocytes and of the serum from similar media with leucocytes, after the colonies had been allowed to grow for 48 hours. Other experiments were made in order to study the action of temperature on serum and cells. A culture medium without

[1] Felton, L. D., *J. Biol. Chem.*, 1921, xlvi, 299.

TABLE I.

Action of Serum from Cultures of Chicken Leucocytes on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
		Without leucocytes (*C*).	With leucocytes (*E*).		Amount of serum.	Amount of hemolysis by serum from cultures.	
						Without leucocytes.	With leucocytes.
					per cent	*per cent*	*per cent*
1	1410	1.50	3.00	2.00	0	0	0
		2.65	3.00				
2	24858–1	2.85	3.84	1.13	0	0	0
		2.74	2.56				
		2.19	4.60				
3	24858–2	3.27	4.10	1.51	0	0	0
		2.82	3.44				
		1.94	3.05				
4	25171	2.36	3.65	1.60	0	0	0
		1.86	3.17				
		2.53	3.97				
		2.94	4.46				
5	25213	2.66	4.28	1.57	0	0	0
		3.40	5.43				
		2.77	3.90				
		2.40	3.35				
6	25391	2.02	3.64	1.45	0	0	0
		3.28	4.00				
		2.59	4.57				
7	28616	3.00	4.94	1.71	0	0	0
		2.57	4.16				
8		3.07	5.41				
	28801	3.19	4.37	1.56	0	0	0
		1.62	3.40		70	0	40
9	28684	2.13	4.09	2.00	50	0	20
					30	0	5
		2.66	4.57		70	0	45
10	28641	4.13	4.29	1.37	50	0	25
					30	0	5

TABLE I—*Concluded.*

Experiment No.	Culture No.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
					Amount of serum.	Amount of hemolysis by serum from cultures.	
		Without leucocytes (*C*).	With leucocytes (*E*).			Without leucocytes.	With leucocytes.
					per cent	*per cent*	*per cent*
11	28770	6.18 3.38	7.00 7.68	1.70	70	0	0
12	29263	4.41 2.57 2.60	9.07 6.30 5.29	2.18	80 40 20	7.00 5.00 2.50	10 10 4
13	28801	3.07 3.19	5.41 4.37	1.56	70	0	0
14	28886	2.38 3.10	1.70 4.66	1.10	70 50 30	0 0 0	40 30 0
15	28926	0	0		80 60 20 10	5.00 2.50 2.50 2.50	40 20 5 2.50
16	29019	0	0		80 60 40 20	10.00 8.00 5.00 2.50	20 15 8 3
17	29162	1.21 3.20 2.26	6.77 5.15 6.38	3.33	80	0	0
18	29400	0	0		80 60 40	60.00 50.00 30.00	75 55 30
19	29059	1.87 3.86 3.38	4.14 6.70 6.80	1.98	80	0	0

leucocytes kept in the refrigerator for 48 hours was compared with a similar medium incubated for the same length of time. The sera from culture media with and without leucocytes, kept in the refrigerator instead of being incubated, were also compared.

1. Serum from Media Incubated for 48 Hours, with and without Leucocytes.—In every case, the colonies grew actively and were still living after 48 hours. In seventeen experiments (Table I), the serum from the media with leucocytes was more favorable to the activity of fibroblasts. The quotient of the rate of growth in serum from leucocyte cultures divided by the rate of growth in serum from media without leucocytes averaged 1.73.

TABLE II.

Action of Serum from Culture Media, Incubated and Refrigerated, on Homologous Fibroblasts and Foreign Erythrocytes.

Experiment No.	Culture No.	Rate of growth in serum from culture media.		Ratio, $\frac{E}{C}$.	Hemolytic action on foreign erythrocytes.		
					Amount of serum.	Amount of hemolysis by serum from culture media.	
		Incubated (*C*).	Refrigerated (*E*).			Incubated.	Refrigerated.
					per cent	*per cent*	*per cent*
1*	29094	1.25	6.53	4.49	80	H	80
		1.68	6.33		20	5	50
					10	0	25
2†	29339	1.04	2.38	3.20	70	15	80
		0.87	3.73		50	8	80
		1.34	4.10		30	3	70
3†	29436	0	0		80	55	75
					40	40	70
					10	10	20

* In Experiment 1, sheep erythrocytes were used.

† In Experiments 2 and 3, rabbit erythrocytes were used.

The hemolytic power of the serum on sheep or rabbit erythrocytes was measured in eleven experiments. In four cases (Table I), both sera were inactive. In seven cases (Table I), the sera from cultures without leucocytes were inactive, while the sera from cultures with leucocytes were hemolytic for sheep or rabbit corpuscles. After the serum had been heated at 56°C., its hemolytic action disappeared completely.

(*a*) *Serum from Culture Media without Leucocytes, Incubated and Refrigerated.*—The effect of heat at 38°C. for 48 hours on media without leucocytes was examined (Table II). The natural hemolytic action of the serum on sheep or rabbit corpuscles decreased after the medium had been incubated, while it remained normal when the medium was kept in the refrigerator. At the same time, the serum from the incubated medium became much less favorable to the growth of chicken fibroblasts (Table II).

TABLE III.

Action of Serum from Cultures of Chicken Leucocytes, Refrigerated, on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
		Without leucocytes (*C*).	With leucocytes (*E*).		Amount of serum.	Amount of hemolysis by serum from cultures.	
						Without leucocytes.	With leucocytes.
					per cent	*per cent*	*per cent*
1	28684	1.33	2.14	1.31	70	40	60
		1.92	2.03		50	20	20
		1.91	2.50		30	5	10
2	28641	5.20	3.83	0.73	70	70	80
					30	10	30
3	28770	4.83	4.82	0.99	50	100	100
		7.93	7.85		30	80	35
4	29263	4.06	4.55	1.91	60	35	35
		2.59	5.16		40	8	20
		2.85	7.47		20	2.5	10
					10	2.5	5

(*b*) *Serum from Culture Media with and without Leucocytes, Refrigerated for 48 Hours.*—In every experiment (Table III), the hemolytic power of the serum from media without leucocytes remained normal, while that from media with leucocytes was slightly increased. At the same time, there was no marked difference in the average rates of growth of fibroblasts in both sera.

2. Serum from Media with and without Leucocytes, to Which a Foreign Protein Was Added.—When 1 per 1,000 casein was added to a culture medium containing no leucocytes, the serum became more inhibiting

for the growth of fibroblasts. It was less toxic when the medium contained only 0.1 per 1,000 casein. A comparison of both sera from cultures of leucocytes with and without 1 per 1,000 casein showed that the presence of casein increased the amount of leucocytic secretion. The juice of the cultures containing casein was always a better medium for homologous fibroblasts than that of the cultures without casein, in spite of the toxic action of the casein (Table IV).

TABLE IV.

Action of Serum from Cultures of Chicken Leucocytes, without and with Casein, on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Casein concentration in medium.	Rate of growth in serum from leucocytic cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
			Without casein (*C*).	With casein (*E*).		Amount of serum.	Amount of hemolysis by serum from leucocytic cultures.	
							Without casein.	With casein.
						per cent	*per cent*	*per cent*
1	28801	1 per 1,000	4.49 4.16 7.90	8.80 7.70 10.50	1.71	70	0	0
2	28886	1 " 1,000	4.81 2.75	5.35 4.23	1.34	70 50 30	40 30 8	40 30 8
3	1410	Dog serum.	1.00	2.50	2.50	0	0	0
4	28988	0.1 per 1,000	2.05 1.54 1.81	2.10 1.32 2.21	1.03	80	0	0
5	29059	0.1 " 1,000	6.71 5.31	6.70 7.25	1.17	80	0	0

The quotient of the rates of growth of fibroblasts in serum from casein media with and without leucocytes was higher than the quotient of the rates of growth of similar cultures containing no casein (Table V). There was little or no difference in the action of the sera when the concentration of the casein in the culture media was 0.1 per 1,000 (Table V).

TABLE V.

Action of Serum from Cultures Containing Casein, without and with Chicken Leucocytes, on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Casein concentration in medium.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
			Without leucocytes (C).	With leucocytes (E).		Amount of serum.	Amount of hemolysis by serum from cultures.	
							Without leucocytes.	With leucocytes.
						per cent	*per cent*	*per cent*
1	28801	1 per 1,000	1.00 0.95 1.13	4.04 4.84 4.63	4.40	70	0	0
2	28886	1 " 1,000	1.00 1.00 1.14	1.94 2.00 2.10	2.03	0	0	0
3	28926	0.5 per 1,000	0	0		80 60 40	5 3.5 2.5	50 30 15
4	29019	0.5 " 1,000	0	0		80 60 20	10 8 2.5	25 20 5
5	29162	0.5 " 1,000	2.88 1.25 1.69	10.00 5.76 8.66	4.39	80	0	0
6	29400	0.5 " 1,000	0	0		80 60 40	60 50 30	75 65 40
7	29059	0.1 " 1,000	6.80 4.81 2.61	12.77 10.35 5.68	2.06	80	0	0
8	29435	0.5 " 1,000	0.65 1.19	2.93 4.90	4.34	0	0	0

No increase of the lytic action on foreign erythrocytes of the serum from culture media containing casein was observed.

IV.

DISCUSSION, SUMMARY, AND CONCLUSIONS.

In the present condition of the technique of cultivation of tissues, the only possible way of studying leucocytic secretions was to grow colonies of leucocytes in a medium of known properties and to examine the modifications of these properties under the influence of the living cells. The method was far from perfect, because the secretions were mixed with serum and accumulated for 48 hours in a medium where they probably underwent partial destruction. But an approximate idea of certain of the qualities of the secretions, although not of their quantity, could be derived from the experiments. In the fluids extracted from the cultures, we attempted to detect the presence of the leucocytic secretions through their physiological effects on homologous and foreign cells. Two kinds of substances were sought, those which act on homologous cells, and those which destroy foreign erythrocytes.

The secretion by leucocytes of substances necessary to the nutrition of other cells was considered as probable long ago. Renaut[2] thought that the main function of the white blood corpuscles was to bring to the fixed cells of the tissues the food material which they need. While the existence of physiological relations between leucocytes and tissue cells could be considered as almost certain, their nature had remained practically unknown. It was probable, however, that the substances secreted by leucocytes were analogous to the growth-activating and unstable substances which are found in embryonic tissues, leucocytes, and certain adult tissues.[3] When connective tissue was aseptically inflamed, or when an aseptic peritoneal exudate contained many leucocytes, aqueous extracts of both connective tissue and peritoneal exudate were found to have acquired the power of stimulating cell proliferation.[4] These experiments showed that leucocytes could bring to the tissues some activating substances. But it remained to be ascertained whether leucocytes, while they are alive, could secrete similar substances either spontaneously or under the stimulus of a foreign factor.

[2] Renaut, J., Traité d'histologie pratique, Paris, 1893, i, pt. 2, 968.
[3] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14; xviii, 287.
[4] Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385.

Leucocytes are supposed to be, as is well know, the origin of the substances which protect the organism against infection.[5] Although the problem of the origin of alexin and antibodies has been investigated by many experimenters, it is not yet completely solved. It was of interest, therefore, to ascertain whether leucocytic secretions could increase the natural hemolytic effect of hen serum on sheep or rabbit erythrocytes, and whether these secretions would become more active under the influence of a foreign protein. The substances which destroy foreign cells are not necessarily different from those which act on homologous cells. The word substance is used for simplicity of description and may be taken as meaning only a given property of an unknown substrate.

A comparison was made of certain properties of sera extracted after 48 hours incubation from media containing leucocytes and from media containing no leucocytes. The serum from the leucocytic cultures was always found to be more favorable to the growth of homologous fibroblasts than the serum from the culture media incubated without leucocytes. The natural hemolytic power of the serum on sheep erythrocytes was found to be increased in about 50 per cent of the experiments.

In other experiments, we found that when two culture media free of cells were placed, one in an incubator at +38°C. and the other in a refrigerator at +5°C. for 48 hours, the serum from the incubated medium partly lost its hemolytic action on sheep or rabbit erythrocytes, while that from the refrigerated medium remained normal; at the same time, the inhibiting action of the incubated medium on homologous fibroblasts had increased very much. This effect of incubation indicates that certain unstable constituents of serum are destroyed by heat. Then the changes found in the properties of the serum from cultures of leucocytes are due to the fraction of the activating substances which has not been destroyed by incubation at 38°C. A quantitative study of the secretions is, therefore, impossible with the present technique, which can furnish only qualitative indications about the substances set free by the leucocytes.

[5] Metchnikoff, E., Immunity in infective diseases, translated by Binnie, F. G., Cambridge, 1905.

We have ascertained also whether a medium containing leucocytes and kept in the refrigerator undergoes any change under the influence of the cells while they are in a condition of latent life. Gabritschewski dishes with and without leucocytes were placed in a refrigerator at a temperature of about +5°C. After 48 hours, the hemolytic power on sheep erythrocytes of the serum from the leucocytic cultures had increased slightly and its inhibiting action on the growth of homologous fibroblasts had decreased. Then certain substances favorable to the growth of homologous cells and toxic for heterologous cells were diffused by the leucocytes into their medium. But the action of these substances was weaker than in the case of the cultures kept in the incubator. This experiment showed that leucocytes under certain conditions diffuse alexin or natural hemolysins which originate from them at the same time as the substances which activate homologous cells. In other experiments, although leucocytes were frozen at −10°C., treated with distilled water, or extracted with saline solution, they did not yield any hemolysin.

To summarize: Leucocytes, cultivated in plasma, always secreted substances which increased the rate of growth of homologous cells. Less frequently, they set free substances which hemolyzed foreign erythrocytes.

The growth-promoting substances are analogous to those contained in embryonic tissues, and probably represent some of the foodstuffs brought to fixed tissue cells by leucocytes. They may possess the function of rejuvenating cells which have ceased to multiply when the cicatrization of a wound or the repair of a fracture requires a resumption of tissue activity.[4] According to this hypothesis, the leucocytes brought to the surface of a wound by the process of inflammation would not only oppose bacterial invasion, but also bring to the tissues the material necessary to cell multiplication. It seems that in some cases regeneration is started by substances brought to the tissues by other cells. Loeb thinks that in *Tubularia*, when endodermic cells gather at the end where a new polyp is about to be formed, the substances given off by these cells are responsible for polyp formation.[6] There may be

[6] Loeb, J., The organism as a whole from a physicochemical viewpoint, New York and London, 1916, 170.

an analogy between this phenomenon and the secretion by leucocytes of growth-activating substances at the surface of a wound.

If we assume that leucocytes *in vivo* set free their secretions in the blood stream, certain variations of the growth-inhibiting action of normal serum can be better understood. The rate of proliferation of homologous fibroblasts is much slower in the serum of an old chicken than in that of a young one.[7] When the serum is heated at 56° and 70°C. for ½ hour, it becomes still more inhibiting.[8] A substance favorable to cell activity has disappeared. It is therefore permissible to suppose that the growth-inhibiting power of serum and its variations are due to the antagonistic action of two substances, one growth-promoting and thermolabile, and the other growth-inhibiting and thermostable, the activating substance being always weaker in its effect than the inhibiting one. We know that activating substances can be extracted from embryonic tissue, from muscle and gland tissues, and from leucocytes of the adult animal, and that they are thermolabile and very unstable. Leucocytic secretions seem to have some of the properties of leucocytic extracts. It is probable that the activating substances which disappear from the heated serum are secreted by leucocytes and other cells. An increase of these secretions, then, would diminish the inhibiting action of serum on homologous fibroblasts. On the contrary, a decrease of the secretions in the serum would increase its inhibiting effect on homologous cells. The strong inhibiting action of serum in old age would be due partly to a reduction in the amount and activity of the substances secreted by leucocytes and tissue cells in the humors of the organism.

Leucocytes also secreted *in vitro* substances which were toxic for foreign cells. Although the results were not constant, the serum appeared to become slightly more hemolytic for sheep or rabbit erythrocytes, under the influence of the leucocytes. The hemolysis of rabbit corpuscles by hen serum is due, according to Hyde,[9] to a complex sensitizer alexin, and not merely to alexin, as Bordet thought.[10]

[7] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.
[8] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 647.
[9] Hyde, R. R., *Am. J. Hyg.*, 1921, i, 358.
[10] Bordet, J., quoted by Hyde,[9] p. 358.

When a foreign protein was added to the culture medium, the leucocytic secretions increased, as was shown by the action on homologous fibroblasts of sera taken from cultures of leucocytes with and without casein. The presence in the medium of the cultures of leucocytes of only 0.1 per 1,000 casein did not markedly modify the action of their serum on the proliferation of fibroblasts. When the concentration of casein in the leucocyte cultures reached 1 per 1,000, the growth of chicken fibroblasts in the serum extracted from the Gabritschewski dishes became more rapid. But there was no parallel increase of the hemolytic action of the serum upon sheep erythrocytes.

We found that chicken serum containing 0.1 per 1,000 casein was barely toxic for homologous fibroblasts, while it became markedly inhibiting when the casein concentration reached 1 per 1,000. Probably, there is a relation between the toxicity of the medium, the increase of leucocytic secretions, and the time of the increase. The change brought about by casein in the equilibrium of the system composed of the cells and their medium determines the secretion by the leucocytes of substances which increase the activity of homologous cells and oppose the inhibiting effect of the foreign proteins. This reaction of the leucocytes is immediate, and may represent the first defense of the organism against a factor which disturbs its equilibrium. Possibly it differs from the specific cell reaction which leads to the production of antibodies. It is known that antibodies develop more slowly. Hemolysins were detected in cultures of bone marrow 4 days after the addition of antigen.[11] The immunization of fibroblasts against foreign proteins has been shown by Fischer[12] to begin after 4 days. If leucocytes behave in the organism as they do *in vitro*, we may assume that before the appearance of antibodies, they respond to the presence of an antigen by setting free growth-promoting substances and possibly alexin. This immediate reaction of the leucocytes against a disturbing factor, and the resulting production of substances which increase the activity of homologous cells, might be partly responsible for the results observed in the treatment of certain diseases by the injection of foreign proteins.

[11] Carrel, A., and Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 287.
[12] Fischer, A., *J. Exp. Med.*, 1922, xxxvi, 535.

It may be concluded that, under the conditions of the experiments:

1. The serum obtained from cultures of leucocytes is less inhibiting for homologous fibroblasts than the serum from media without leucocytes. In some experiments, its hemolytic action on sheep or rabbit erythrocytes is also increased.

2. The addition of casein to leucocytic cultures brings about a decrease in the inhibiting effect of the serum on homologous fibroblasts.

3. The increase in the activity of homologous fibroblasts in serum obtained from leucocytic cultures is probably due to growth-promoting substances secreted by the leucocytes. The presence of a foreign protein under certain conditions determines a more abundant leucocytic secretion.

THE LOSS OF CIRCULATING ERYTHROCYTES IN CERTAIN TYPES OF EXPERIMENTAL PNEUMONIA.

By CHAUNCEY D. LEAKE and THOMAS K. BROWN.

(*From the Physiological and Bacteriological Laboratories of the University of Wisconsin, Madison.*)

(Received for publication, June 23, 1922.)

During the course of a prolonged investigation of experimental pneumonia, a red blood cell count was made as a routine procedure. Our first thought was that there would be a compensatory rise in the number of erythrocytes to counteract the effect of interference with blood oxidative processes in the lung as a result of the pneumonic lesions. We found otherwise.

In the work on pneumonia some 60 dogs were employed. Of these, 36 were studied for changes taking place in the blood.

1. Inoculated with *B. bronchisepticus*, 14 animals (Nos. 1 to 14).
   8 satisfactory: 2 killed, 3 died, 3 recovered.
   6 unsatisfactory: 1 died too soon, 5 ineffective inoculations.

2. Inoculated with *Streptococcus hæmolyticus*, 3 animals (Nos. 16, 17, and 18).
   1 satisfactory, recovered.
   2 unsatisfactory, ineffective inoculations.

3. Inoculated with *Streptococcus viridans*, 2 animals (Nos. 20 and 24).
   1 satisfactory, recovered.
   1 unsatisfactory, ineffective inoculation.

4. Inoculated with Pneumococcus Type I, 1 animal (No. 21).
   1 unsatisfactory, ineffective inoculation.

5. Inoculated with *B. influenzæ*, 1 animal (No. 22).
   1 satisfactory, recovered.

6. Inoculated with *B. mucosus capsulatus*, 39 animals (Nos. 15, 19, 23, and 25 to 60).
   25 satisfactory: 1 killed, 16 died, 8 recovered.
   14 unsatisfactory: 7 died too soon, 2 inadequately observed, 5 ineffective inoculations.

It is to be noted that 46 of the dogs were successfully inoculated and showed clinical symptoms of pneumonia. Of this number, eight died within 18 hours of inoculation, so that it was impossible to obtain observations upon them.

Inoculations were made by tracheal insufflation (1). Fifteen to twenty cultures of a 24 hour growth of the organisms on Löffler's slants were suspended in 10 cc. of Ringer's solution. The animal was anesthetized and a small rubber tube was passed down the trachea through the mouth to the tracheal bifurcation. The suspended organisms were injected through this tube by syringe, and were immediately followed by 10 cc. of Ringer's solution, and then by 10 cc. of air. The purpose of this was to blow the trachea clean, and to spread the organisms as far as possible into the bronchial tree. The dog was kept under ether for 5 minutes longer to prevent the coughing up of any of the organisms.

In the successfully inoculated and observed animals, lung lesions were followed by fluoroscope and x-ray examination; 20 cc. of blood were drawn daily until death or recovery, for the different blood determinations, and lung ventilation was daily recorded by means of a body plethysmograph. A report on these observations will be made at some later time. The blood was drawn from the femoral artery into an oxalated syringe through the intact skin, with suitable aseptic precautions and means to prevent hemorrhage. In this manner circulating blood was obtained for the counts, obviating any chance of securing blood diluted with serum as in the cases in which blood is drawn from ear punctures. The dilution resulting from washing the syringe with sterile saturated potassium oxalate solution before drawing the blood may be considered negligible.

Erythrocyte counts in duplicate, with the American standard hemocytometers with Levy counting chambers certified by the Bureau of Standards, were made on thirty of the thirty-six satisfactory dogs. In all these animals a marked decrease in red cells was noted as the disease progressed. If the animal recovered, the tendency to return to a normal count was not pronounced or in proportion to the speed of recovery. A typical animal, for instance, inoculated with *Bacillus bronchisepticus* (No. 6) had a normal erythrocyte count of 6.6 million per c.mm. 2 days after inoculation, with both

lungs diffusely shaded, the count was 5.2 million. 2 days later, with the right lung densely shaded, and the dog very sick, the count had fallen to 4.4 million, and 3 days later, just before the animal died, the count was 3.6 million. In a dog which recovered from a *Bacillus bronchisepticus* infection (No. 9), the normal erythrocyte count was 6.8 million; 3 days after inoculation, with marked distemper and the left lung diffusely shaded, the count was 5.1 million; the next day, with both lungs cloudy, the count was 4.5 million; on the following day, however, with the lungs clearing and the animal's physical condition markedly improved, the count was 4.2 million; and 2 days later, with the lungs clear, the count had risen only to 5.2 million. The same general effect was noted in the animals successfully inoculated with streptococcus and influenza organisms. Typical erythrocytic effects after inoculation with *Bacillus mucosus capsulatus* are shown in Table I.

The uniformity of the results raised the question as to whether there was an actual loss of circulating erythrocytes, or whether the blood was merely diluting. Accordingly, blood volume determinations by means of the method of Keith, Rowntree, and Geraghty (2), hemoglobin determinations by Palmer's method (3), and hematocrit determinations were made on a small number of the dogs successfully inoculated with *Bacillus mucosus capsulatus*. The results are shown in Table I. Blood volume, within the limits of error of the method, remained constant; the hemoglobin fell approximately in proportion to the fall in the number of red cells, and there was a marked increase in plasma with a corresponding fall in the mass of formed elements. These considerations indicate an actual loss of erythrocytes in the effective circulation, without a compensatory regeneration.

Histological search through the tissues of the various organs after death revealed the red cells in different stages of degeneration in large quantities in the capillaries and tissue spaces of the lungs, liver, spleen, and intestinal walls. The capillaries in all these organs were fairly gorged with red cells, and even in the capillary network of the glomeruli of the kidney there were more red cells than in normal animals. The tissue spaces did not contain erythrocytes except in the lungs and spleen. The latter held a great mass of red cells in degeneration.

The literature on the subject of the variations in the number of erythrocytes in pneumonia is scanty. Cabot (4) notes clinically that there may be a slight anemia following the crisis in pneumonia, and cites Grawitz as stating that the post-critical relaxation of peripheral vessels causes a dilution of the blood with an apparent loss of red cells. Rosenow (5) cites Boeckman as saying that the number of erythrocytes is proportional to the degree of temperature in pneumonia, and himself declares that there is scarcely any infection in which the production of

TABLE I.

Effect of Inoculation with B. mucosus capsulatus on Erythrocytes in Dogs.

Dog No.	Date.	Weight.	Erythrocytes per c.mm.	Hemoglobin.	Blood volume.	Plasma.	Corpuscles.
	1921	*kg.*	*million*	*per cent*	*cc.*	*per cent*	*per cent*
49	Aug. 4	9.1	7.2	125		54	46
Died.	" 5	9.0	6.1	105		58	42
51	" 15	6.6	7.3	115	590	54	46
Recovered.	" 16	6.4	6.0	101	610	59	41
52	" 16	9.6	7.5	127	1,090	44	56
	" 17	9.5	5.6	104	1,060	51	49
	" 18	9.4	5.5	101	1,030	52	48
Recovered.	" 19	9.5	6.3	110	960	52	48
57	Nov. 3	9.2	7.2	102		54	46
	" 5	9.1	7.0	101	980	54	46
Died.	" 7	8.0	4.2	53	920	69	31
59	" 17	5.8	6.3	105	600	59	41
	" 21	5.6	5.3	90	610	63	37
Died.	" 22	5.1	4.2	76		65	35
60	" 23	8.0	7.2	97		42	58
Died.	" 25	6.8	5.3	80		53	47

The figures for the 1st day in each case are normal, before inoculation.

anemia is so rapid as in pneumonia. Burnett (6), however, in discussing croupous pneumonia in horses, notes that the number of erythrocytes does not greatly change, except sometimes to increase slightly because of the concentration of the blood due to loss of fluid.

We observed that three of the animals in which pneumonia had been induced, although they were in fair condition while lying in

their own chosen curled up position, quickly became dyspneic and cyanotic when placed on their backs.

Erythrocyte counts, blood volume, hemoglobin, and hematocrit determinations were made upon one control animal. This dog lost approximately the same amount of blood as the others, and was subjected to the same manipulative, fluoroscopic, and x-ray procedures, with the exception that it was not inoculated with any organism. The results are shown in Table II. It may be seen that such variations as occurred in the determinations were insignificant.

TABLE II.

Control Dog.

Date.	Weight.	Erythrocytes per c.mm.	Hemoglobin.	Blood volume.	Plasma.	Corpuscles.
1921	*kg.*	*million*	*per cent*	*cc.*	*per cent*	*per cent*
Oct. 7	7.2	6.2	98	800	51	49
" 10	7.3	6.4	100	790	52	48
" 14	7.2	6.1	97	740	54	46

SUMMARY.

Erythrocyte counts, blood volume, hemoglobin, and hematocrit determinations on a series of dogs suffering from experimental pneumonia induced by the tracheal insufflation of *Bacillus bronchisepticus* and *Bacillus mucosus capsulatus* indicate that in this condition there is an actual loss of circulating erythrocytes, without compensatory regeneration.

Postmortem examination of the organs of these animals revealed red cells in various stages of degeneration in the capillaries and tissue spaces of the lungs, liver, spleen, and intestinal walls.

We wish to thank Professor W. J. Meek and Professor P. F. Clark for encouragement in this work.

BIBLIOGRAPHY.

1. Lamar, R. V., and Meltzer, S. J., *Proc. Soc. Exp. Biol. and Med.*, 1909–10, vii, 102.
2. Keith, N. M., Rowntree, L. G., and Geraghty, J. T., *Arch. Int. Med.*, 1915, xvi, 547.

3. Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119.
4. Cabot, R. C., A guide to the clinical examination of the blood for diagnostic purposes, New York, 3rd edition, 1898, 185.
5. Rosenow, E. C., *J. Am. Med. Assn.*, 1905, xliv, 871.
6. Burnett, S. H., The clinical pathology of the blood of domesticated animals, New York, 2nd edition, 1917,101.

THE SUPRAVITAL STAINING OF VACCINE BODIES.

By EDMUND V. COWDRY, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 58 and 59.

(Received for publication, June 26, 1922.)

INTRODUCTION.

To determine the nature of the conspicuous cell granulations found in the lesions of vaccinia is a problem in cytology of unusual interest which has continued to hold the attention of investigators.[1] They seem to represent the visible expression of a fundamental cellular reaction. From the historical point of view three lines of thought may be recognized in the development of our present knowledge of the subject, which, in its broader aspects, cannot well be considered apart from other diseases of unknown etiology.

1. On their discovery, the granulations were first named after their observers, Guarnieri's bodies in vaccinia and variola, Negri's bodies in hydrophobia, von Prowazek's bodies in trachoma, Mallory's bodies in scarlet fever, etc. Influenced, perhaps, by contemporaneous studies in malaria and other protozoal diseases, investigators have described complicated life histories based partly upon direct observations and partly upon analogy. The granules were hailed more or less tentatively as the actual parasitic causes of the diseases, were grouped among the protozoa, and were given generic and specific names, of which *Cytoryctes vaccinia* in vaccinia, and *Neurocytes hydrophobiæ* in hydrophobia will serve as examples.

2. But closer and repeated scrutiny of the life histories did not turn out to be reassuring, and led investigators to question whether a real step had been made in advance. A reaction set in and several papers appeared showing most clearly that in some cases at least the granules are not cell parasites but deposits of pathologic nature. By common consent the protozoal terminology was abandoned and the names of the discoverers reinstated.

3. Faced by this association of more or less specific granulations with certain infectious diseases, the organisms of which have not been seen and remain almost

[1] The early literature is reviewed by Councilman, Magrath, and Brinckerhoff (1904), by Calkins (1904), by Paschen (1911), and by von Prowazek (1912).

wholly unknown, von Prowazek (1907) offered a compromise hypothesis according to which the cytoplasmic granules are of dual nature, consisting of microorganisms embedded in a substance of nucleolar origin produced by the cell in response to their action. Von Prowazek has proposed that these organisms be grouped in a new class which he has called Chlamydozoa—literally, mantle animals—to indicate this habit of being clothed in a mantle of cellular material. In his opinion they are characteristically intracellular parasites and differ from bacteria in their method of multiplication as well as in other respects. He conceived their life history to be somewhat as follows: In the stage of elementary corpuscles they are extracellular and filterable; soon after penetration through the cell membrane they may be detected in the form of tiny initial bodies. These become coated with cellular material, grow, mature, and break down with the liberation of large numbers of the original elementary corpuscles, which, in turn, enter and infect neighboring cells with a repetition of the process.

Lipschütz (1912) has devised the term Strongyloplasmata to indicate organisms endowed with much the same attributes. He groups (1921) under this heading the viruses of molluscum contagiosum, bird-pox, sheep-pox, vaccinia, scarlet fever, verruga peruviana, herpes (zoster, genitalis, and febrilis), varicella, variola, and paravaccinia, and is supported in his contention regarding vaccinia by Gins (1922) and other recent investigators.

It seems almost to have become the fashion to classify organisms about which very little is known in one or the other of these two groups. For example, during the war the ravages of typhus and trench fever demanded very close attention in both camps, which contributed directly to our knowledge of organisms which appear to be intimately concerned in the etiology not only of these two diseases but also of Rocky Mountain spotted fever and perhaps of tsutsugamushi disease, or Japanese river sickness. They have been called *Rickettsia* in honor of Ricketts who lost his life in Mexico in 1910 while investigating their behavior in typhus, but the term is unfortunate because it carries with it the suggestion that the organisms differ from ordinary bacteria and form a group exhibiting certain characteristics in common. Although it has not been possible to prove that during some phase of their life cycle they are of dual nature (consisting in part of cellular substance), they have been referred to the Chlamydozoa by da Rocha-Lima (1916), Jungmann (1919), and others.

Looking through the recent literature one finds the opinion repeatedly expressed that real advances in our knowledge of vaccinia will only be made with the help of new methods of technique.

Steinhardt and his associates (1913, 1914) and Harde (1921) have invoked the methods of tissue culture and have succeeded in obtaining a definite multiplication of the virus *in vitro* but have not been able to concentrate it greatly or to determine its relation to vaccine bodies.

What appears to be a new avenue of approach to the study of infectious diseases of unknown etiology is contributed by MacCallum and Oppenheimer (1922), who so concentrated the virus of vaccinia, through the method of differential centrifugation, that they have been able to observe directly certain granules, which may be the infective agents, under dark-field illumination and with ordinary powers of the microscope. "They occur singly or in small groups, or sometimes in tiny beaded chains. . . . . They stain faintly with Löffler's methylene blue, are gram negative, do not stain with neutral red or trypan blue, stain blue or red with Wright's stain, and take a fairly deep stain with carbolfuchsin." The authors believe that: "these granules are apparently identical with those seen in tissues and smears from vaccinia and smallpox by Prowazek, Paschen, Hallenberger, and others." A similar method is advised by Ségal (1922) for concentrating the virus of typhus fever.

A large supply[2] of tissues from vaccinated rabbits in various stages of the reaction has given me an opportunity to experiment widely in technique and thus to make a cytologic study of the cornea with three definite objects in view; namely, (1) to trace the origin of the material of which the vaccine bodies are constituted, (2) to ascertain whether it is in part of chlamydozoal nature, and (3) to determine how closely it is related to the granules described by MacCallum and Oppenheimer in vaccine lymph.

OBSERVATIONS.

If the edge of a sharp scalpel is passed over a typical corneal lesion and the cells thus obtained are immersed in physiological salt solution and examined unstained under direct illumination, they may be seen to contain a great variety of materials some of which are not visible in control preparations of normal corneas. These may be partly resolved into the following groups.

1. Vaccine bodies, which are easily recognized by their relatively large size, low refractive index, and their tendency to be closely associated with the nuclei. Two of them are illustrated in Fig. 18, which

[2] This material was kindly placed at my disposal by Dr. Noguchi. It consisted of two rabbits 1 day after infection with vaccine prepared by a special method (Noguchi, H., Pure cultivation *in vivo* of vaccine virus free from bacteria, *J. Exp. Med.*, 1915, xxi, 539) by which it is freed from bacterial contamination, two 2 days after infection, three 3 days, two 4 days, one 5 days, three 6 days, one 7 days, one 8 days, one 9 days, and three controls, making nineteen in all.

should be compared with Fig. 1 representing a similar cell from an unvaccinated cornea.

2. Highly refractile spherical droplets which are usually gathered together in clumps and are probably of lipoidal nature.

3. A few scattered, rod-shaped mitochondria which may be definitely identified by the addition of a little Janus green, which colors them specifically when used in sufficient dilution (Fig. 20).

4. Large masses of coccal and bacillary shaped bodies of low refractive index, as represented in Fig. 19. Their occurrence is variable and does not seem to parallel the vaccine reaction. No independent motility could be established. They may be temporary phase granules owing their appearance to some alteration in osmotic conditions, or they may represent an accidental contamination with bacteria.

5. Large, highly refractile droplets of neutral fat which vary considerably in number, resist the solvent action of 1.5 per cent acetic acid drawn under the cover-glass, and are blackened by treatment with osmic acid.

Through the addition of brilliant cresyl blue 2 B[3] to the physiological saline solution in a concentration of about 1:25,000, the vaccine bodies are almost instantly stained a color varying from pink to dark blue (Figs. 3 to 17) and the stages in their formation may easily be studied. Why von Prowazek (1912) failed to obtain a definite color reaction with this dye I am unable to suggest, unless the sample used was of different constitution or employed in a different way.

As a control measure brilliant cresyl blue was applied to testicle lesions with the same result. It was found that the same material will stain a brick-red color with neutral red and that Janus green will color it when sufficiently concentrated. Unfortunately, staining with these dyes is largely a process of selective adsorption, so that it does not convey any information regarding the chemical constitution of the material stained. But it is, perhaps, not without significance, regarding the nature of vaccine bodies, that the dyes are true solutions commonly used for the study of cellular constituents, and that they have achieved no popularity as an aid to the study of microorganisms.

[3] For the manufacture of brilliant cresyl blue 2 B see Schultz (Schultz, G., Farbstofftabellen, Berlin, 1914).

Although they are relatively non-toxic (Hogue, 1922), these methods of supravital staining are open to serious question unless very carefully controlled. Since the cells are slowly dying, not through any toxic action of the stain but on account of traumatism and unfavorable environmental conditions, it is important not to prolong observations more than 30 minutes after the cells are removed from the cornea; certainly the moment that they show evidences of shrinkage, or of the adsorption of fluid with the release of molecular bombardment causing brownian movement, or of coloration of the nuclei, the preparations should be discarded.

The fragmentary appearance of the smaller vaccine bodies precludes any possibility of confusion with the segregation apparatus, originally described by Renaut, which is brilliantly colored by this technique in certain blood and connective tissue cells in which it takes the form of clusters of rounded droplets. Another point of distinction is that in smallpox similar bodies are found within the nuclei. A high development of this apparatus is suggested by Simpson (1922) as a criterion for the identification of monocytes.

With the fact in mind that in some respects the study of fixed tissues is less likely to be misleading because in them all vital activities are simultaneously arrested in some definite physiologic phase, every effort has been made to extend and to correlate both lines of inquiry. Many fixatives,[4] stains,[5] and microchemical methods[6] have been applied to infected and normal cornea and testicles.[7]

[4] Zenker's fluid with and without acetic acid, formalin, Regaud's fluid, Giemsa's sublimate mixture, acetic-sublimate, absolute alcohol, Altmann's fluid, Bensley's acetic-osmic-bichromate mixture, osmic acid in various concentrations, da Fano's modification of Cajal's silver method, etc., were used. In addition, "*Klatsch*" preparations, made as suggested by Ewing, were fixed by heat, by the vapor of formalin and osmium, by absolute alcohol, and in other ways.

[5] The stains used were Borrel's, Giemsa's, Löffler's methylene blue, Mallory's eosin and methylene blue, Wright's stain, iron-hematoxylin, Pappenheim's pyronine and methyl green, carbolfuchsin-acid violet, aniline fuchsin-methyl green, Herxheimer's Scharlach R, etc.

[6] Mayer's muchematein for mucus, the Macallum-Bensley iron reaction as suggested by Nicholson, Millon's reagent, and Schultze's oxidase reaction.

[7] I am also indebted to Dr. Noguchi for allowing me to study his extensive series of stained preparations of vaccinia in rabbits and in calves.

In corneal cells supravitally colored with brilliant cresyl blue, the irregularity in the form and distribution of the material from which the vaccine bodies are built is most noticeable. It varies from the very smallest aggregates, probably even beyond the limits of microscopic visibility, which do not concentrate the dye, and which cannot be resolved with the aid of the best lenses (Fig. 3), to roughly spherical masses upwards of 5 μ in diameter (Figs. 11, 12, and 15). This variability is exhibited by neighboring cells in almost any active stage of the process from 2 to about 9 days after vaccination. This is shown with equal clearness in fixed tissues (Figs. 21 to 45). After search through a number of preparations, or even in a single section, masses of material may be selected which correspond in size and shape to the elementary corpuscles, initial bodies, and reaction bodies described in the literature;[8] but considered all in all, so haphazard is their morphology that it is difficult to satisfy oneself of the existence of a progressive series of morphological changes indicative of independent growth other than a simple increase in size of the aggregates through the accretion of more and more material. It is equally difficult to make out a breaking down of the large masses with a liberation of the original tiny fragments. Fig. 35 may represent a dispersal of elementary corpuscles, or it may represent a stage in the condensation of small bodies into a larger mass, depending upon which way it is interpreted.

That we are in fact dealing with a tremendous development of material which does not owe its ultimate origin to an infective process is shown by the demonstration of traces of a similar substance when the same technique is applied to the unvaccinated cornea. Its substantial similarity, as observed in normal cells supravitally colored with brilliant cresyl blue and in normal cells after fixation in Zenker's fluid (less acetic acid) and staining with Giemsa's stain may be seen by comparing Figs. 2 and 22. There is also a close parallelism between its increase in amount, under the influence of vaccination, as one follows it in supravitally stained cells and in fixed tissues. When colored with a relatively strong solution of Janus green

[8] A close comparison may be drawn with Figs. 2 to 6 in the section on vaccine in von Prowazek's handbook to protozoa.

(1:10,000) the material changes to pink and bleaches with the formation of the color base and leuco base on reduction at the same time as do the other cytoplasmic constituents. After fixation in Altmann's fluid, and other mixtures containing osmium, it is, like the chromidial substance in gland cells, somewhat more diffuse. It would be unsafe to say that it occurs in definite aggregates in normal living cells *in vivo*, for the technique of its demonstration in living cells and in fixed tissues may easily, and probably does, produce artificial changes in the colloidal substratum of the cytoplasm. In other words, the material, as we see it, is the expression of the treatment we accord the cells; its occurrence in small quantities is not dependent upon vaccination.

Owing to their larger size, the vaccine bodies lend themselves more easily to microchemical analysis than does this primordial material, but beyond the statement that they are in part, at least, of protein nature, little may be said with certainty. Their resistance to tryptic and peptic digestion has been noted by von Prowazek (1912). Attempts to secure a definitely positive reaction to Millon's reagent and to the Bensley-Macallum test for iron have been unavailing, but my failure may be due to technical difficulties. Neither do they respond to Schultze's reaction for oxidase granules. They are almost immediately dissolved when a 1.5 per cent solution of acetic acid is drawn under the cover-glass. Since vaccine bodies which are apparently similar develop in the interstitial cells of the testis, and are recorded in the literature in many other locations, it follows that the original material is to be regarded as a fundamental constituent of protoplasm.

The phagocytosis of entire leucocytes and of other corneal cells is a phenomenon of common occurrence (Figs. 16, 39, and 40), but I think that the vaccine bodies do not arise through the digestion of red blood cells,—an old idea which Woodcock (1921) has recently reaffirmed,—because I have not been able to distinguish any traces of hemoglobin coloration in freshly teased corneal cells. The test for masked iron, on which he places so much reliance, can hardly be expected to differentiate between iron-containing compounds of hematogenous and nuclear origin. Moreover, the properties of the bodies, as detailed above, and the occurrence of minute deposits of similar material in unvaccinated corneas are not easily reconciled with this view. The

theory that they are formed through the phagocytosis of leucocytic fragments breaks down in the absence of data regarding consecutive stages in the process. In the normal cornea there is little or no breaking down of leucocytes, and after vaccination the leucocytic increase is somewhat variable and does not closely parallel the development of definitive vaccine bodies. Neither are vaccine bodies more abundant, or larger, near foci of leucocytic destruction.

It seems more likely that the material of which they are composed is endogenous. That it is not related to mitochondria is evident from the application of the Janus green reaction (Fig. 20) and from the general appearance of mitochondria in fixed preparations of the cornea (Fig. 45). Although I have not been successful in obtaining satisfactory slides of reticular material (Golgi apparatus) in the cornea, silver preparations of vaccinated testicles indicate that it does not constitute a source of confusion. In consideration of Ewing's (1904–05) studies with "*Klatsch*" preparations, a nuclear origin is strongly indicated, some investigators going so far as to suggest that in studying vaccine bodies we are dealing wholly or in part with extruded nucleoli. The avidity with which the vaccine bodies take up basic dyes and their topographic association with the nuclei are worthy of comment in this connection. The failure of brilliant cresyl blue to bring to light similar material within the nuclei (Figs. 2 to 17) does not militate against this conception because the dye is not able, in dilute solution and in so short a space of time, to penetrate through the nuclear membranes. At present it seems only safe to conclude that the material may be in part of nuclear origin. It would be very difficult to prove that it is wholly so.

When, under the stimulus of vaccination, the material is deposited in fairly large masses, it shows evidences of heterogeneity in both living cells and in fixed tissues. Supravital staining with brilliant cresyl blue reveals the presence of blue-staining droplets, or granules, within the larger pink-staining masses. The blue droplets vary in size, but only in rare instances are they as large as the pink-staining material (Fig. 17). They are also of less frequent occurrence. In some cases the vaccine bodies contain masses of high refractive index and irregular shape, which do not stain and which appear to be of less fluid consistency. Occasionally these occur free in the cytoplasm.

Fixed preparations also furnish evidence of corresponding diversity in composition. Similar droplets are visible in the vaccine bodies after a variety of fixations, of which Zenker, acetic-sublimate, formalin, and Regaud may be mentioned. They remain uncolored after treatment with the Borrel combination (as suggested by Calkins) Mallory's eosin-methylene blue, carbolfuchsin-acid violet, and other dyes. The chromatophobe, refractile masses, above mentioned, have not been seen after fixation. With Wright's stain after Zenker fixation, a rough gradation may be established between small irregular vaccine bodies which are colored dark blue and certain larger ones in shades of gray which take a pink or a light robin's egg blue color, as represented in Figs. 27 and 36. Sometimes these larger masses are homogeneous, or they may be surrounded with a fringe of dark blue material, as indicated in Fig. 36. Staining with aniline methyl violet, treating with Gram's iodine solution, and differentiating in 95 per cent alcohol discloses a similar heterogeneity, as shown in Fig. 40. Giemsa's stain (Fig. 41) brings to light much the same effect, it being possible to modify at will the color values from dark blue to reddish blue by inserting traces of colophonium in the alcohol of differentiation, as suggested by Wolbach, Todd, and Palfrey (1922) for the demonstration of *Rickettsia*.

From these observations I infer that the material is labile, and that, as the larger vaccine bodies are formed, it undergoes some dissociation or takes up substances from the cytoplasm so that it loses its original optical homogeneity; but this change can hardly be regarded as suggestive of the presence in it of independent microorganisms in the hypothetical stage of initial bodies.

Neither is the physical behavior of the material suggestive of the presence in it of microorganisms. In cells which are still living, it has not been possible to detect evidence of independent motility. The smallest bodies show a tendency to be distributed fairly uniformly throughout the cytoplasm and the larger ones to be congregated near the nuclear membrane. In common with other cytoplasmic materials its form and position are determined by cellular stresses, by electrical charge, and by other physical and chemical forces. For example, in the depth of the lesion, where the cells are often pressed together laterally, the deposits of material fre-

quently assume an elongated shape and become disposed parallel to the long axis of the cell. When the shape of the cell is altered through manipulation, the shape assumed by the material also changes and in some cases it seems to flow upon the surface of the nucleus, as is shown in Figs. 5 and 9, but the movement exhibited is in no sense independent or ameboid. That the material is very fluid in consistency and exhibits but little internal organization requires no further proof. The larger masses are perhaps more fluid than the smaller, more angular fragments, and have rounded up into spheres by virtue of the law of least surfaces.

In this respect also the study of permanent preparations is confirmatory. The habit the material shows of conforming in shape to that of the cell under pressure is illustrated in Fig. 28—its flowing motion in Fig. 30. As in the living cells, so also here the larger masses are spherical, but they show another property in their tendency to lodge in infoldings of the nuclear membrane. Sometimes they appear to press into the nucleus from opposite sides (Fig. 33), while in other cases they are found in deep invaginations of its surface, which may be single or multiple, and which convey the impression, when cut in transverse section, that the vaccine bodies are actually intranuclear, as represented in Figs. 34, 38, and 40. But pictures of this kind are not so easily interpreted. Whether the vaccine bodies actively indent the nuclei by virtue of being of more solid consistency, or whether they flow into depressions produced in the yielding surfaces of the nuclei through shrinkage, it is difficult to say. It seems clear, however, that the indentations are artificial since they are not to be seen in living cells (compare Figs. 3 to 20).

Considerable attention has been devoted in the literature to the areas of clear cytoplasm, or halos, which surround the vaccine bodies (Figs. 23 to 35). Investigators have called to mind approximately similar halos as accompaniments of bacteria in tissues. The fact that they are not visible in the case of vaccine bodies supravitally stained with brilliant cresyl blue is not very helpful, because neither do I find the bacterial halos in insect material. Nevertheless, it can hardly be denied that their appearance is strongly suggestive of shrinkage at interfaces between fluids of different consistency and composition. That their extent is in some measure proportional to the size of the

vaccine bodies will be seen by comparing Figs. 23 and 27 with Fig. 33. The presence of slight halos about the nuclei (Figs. 25 and 26), though probably produced in the same way would hardly be taken to mean that the nuclei behave like independent microorganisms.

In discussing the significance of cell inclusions in scarlet fever, Mallory (1903–04) expressed the opinion that:

> "In order to prove that a series of bodies are stages in the developmental cycle of a protozoon we are dependent on three things, namely, ameboid motion, definite and characteristic morphology of the various bodies found, and the demonstration that the bodies go through a progressive series of changes or developmental cycle resulting in increase in size followed by division and the re-formation of the small bodies from which the series started."

From the foregoing account it is apparent that I have not been able to fulfill any of these conditions.

The problem is complicated by the fact that well formed vaccine bodies have been repeatedly recorded outside the cells. In some of my preparations certain bodies, bearing a resemblance, perhaps entirely superficial, to intracellular vaccine bodies, are to be seen in the fibrous tissue beneath the epithelial layer of the cornea. Whether they have been formed *in loco* or have escaped from degenerating epithelial cells or leucocytes cannot be definitely stated in the absence of specific criteria for their identification. The chlamydozoal hypothesis does not provide for their development apart from the cells. Attention has already been directed to the fact that according to this theory they are said to be of dual nature, consisting of intracellular parasites in the form of initial bodies cloaked in a mantle of material elaborated by the cell. This assertion belongs to the category of statements which are easy to make and exceedingly difficult either to prove or to disprove. If the granules described by MacCallum and Oppenheimer are in fact the infective agents, and, if they maintain throughout their life cycle the morphological and tinctorial properties ascribed to them, then it appears to be unlikely that the virus is contained within the vaccine bodies. But, should the virus be beyond the limits of visibility with the best lenses, then its relation to the vaccine bodies is not easily determined.

While I have not made a cytological study of other diseases attributed to chlamydozoal organisms, a careful review of the literature does indicate, that, as far as the nature of the visible cytoplasmic granulations is concerned, their inclusion in a single category is scarcely warranted. Particularly does this appear to be the case in trachoma (Solovief, 1921).

In comparing the properties of the granules described by MacCallum and Oppenheimer in vaccine lymph with those of typical intracellular vaccine bodies, several points of similarity and of dissimilarity may be noted. (*a*) Like the granules of vaccine lymph the vaccine bodies are not specifically colored with trypan blue, and are colored various shades of blue and red with Wright's stain. They apparently surpass the granules of vaccine lymph in the intensity of their coloration with Löffler's methylene blue and especially with carbolfuchsin. (*b*) Unlike them, they are Gram-positive in the sense that they resist decoloration with alcohol after staining with aniline methyl violet and treatment with Gram's iodine. They are, moreover, intensely stained with neutral red. But still more striking is the difference in morphology. Instead of being characterized by their uniformity, the vaccine bodies are extremely variable. They bear no resemblance to tiny beaded chains of streptococci, as do the granules in vaccine lymph described by MacCallum and Oppenheimer.

To the claim that these reactions are not specific and mean but little one way or the other,—which is freely admitted,—and, further, that one would not expect the granules in vaccine lymph to maintain their original uniformity of morphology after penetration into the cell where they find themselves under entirely different surroundings, it is but natural to advance the observation of the existence of traces of material similar to the vaccine bodies in unvaccinated corneal cells. If the granules which these investigators have described are in truth the infective agents, they cannot be taken to correspond with material seen in uninfected cells, however imperfect our knowledge of its chemical constitution may be.

Since, however, the report of MacCallum and Oppenheimer is of a preliminary nature, many important details being reserved for a subsequent contribution, it seemed desirable to obtain lymph from the

same source and to repeat their work in order to make a more direct comparison with intracellular vaccine bodies.[9]

Bearing in mind the results obtained by MacCallum and Oppenheimer, I took about 5 cc. of this lymph, centrifuged it at high speed for 1 hour, drew off the turbid supernatant fluid,—which in their experiments contained the virus,—and divided it into six portions. Through the addition of varying amounts of glycerol to samples of Locke's solution, solutions were made up to the following specific gravities, determined by hydrometer: 1.11, 1.12, 1.13, 1.14, 1.15, and 1.16. One of these fluids was added to each of the six portions and centrifuged. Although the fresh supernatant fluid was examined in each case, no aggregates suggestive of organisms were found. In attempting to prepare permanent specimens, the relatively large amount of glycerol constituted a considerable handicap. Fixation in formalin vapor and absolute alcohol proved unsuccessful, but after prolonged treatment of smears with the vapor of a slightly warmed 2 per cent solution of osmic acid followed by desiccation, it was possible to color certain granulations intensely blue with Giemsa's stain in the lymph which had been centrifuged in a fluid of 1.14 specific gravity. It will be recalled that MacCallum and Oppenheimer believe the specific gravity of the virus to be about 1.12 or 1.13.

On close examination these granules were seen to be roughly spherical and to vary in diameter from 0.2 to 0.4 μ. Sometimes the larger ones are slightly angular. They occur free or embedded in a loose coagulum which stains a light lilac color. Although they are quite frequently grouped end to end in series, the individual segments are not always of the same size and rarely exceed four or five in number. The same granules are tinted gray, not black, with osmic acid. Judging from the brief description by MacCallum and Oppenheimer their appearance is only remotely suggestive of the more uniform bodies which they found.

[9] I am again indebted to Dr. Noguchi for his kindness in giving me a large supply of vaccine lymph which he obtained from the H. K. Mulford Laboratories through the courtesy of Dr. W. F. Elgin.

CONCLUSIONS.

Vaccine bodies in living corneal cells may be specifically stained by the addition of a small quantity of brilliant cresyl blue 2 B to the physiological salt solution in which they are being observed. Their appearance by this method (Figs. 3 to 17) corresponds with that seen in fixed preparations (Figs. 22 to 42). Both lines of study reveal the existence of traces of similar material in unvaccinated corneal cells. As this increases in amount during the reaction, it behaves like an integral, cytoplasmic constituent of fluid consistency and shows no evidence of being endowed with any measure of independent vitality. The low grade of structural differentiation which it does exhibit, in living cells as well as in fixed tissues, is not suggestive of the presence within it of independent microorganisms. The material differs radically in its morphology and microchemical reactions from the granules observed by MacCallum and Oppenheimer in vaccine lymph.

BIBLIOGRAPHY.

Calkins, G. N., The life history of *Cytoryctes variolæ*, Guarnieri, *J. Med. Research*, 1904, xi, 136.

Councilman, W. T., Magrath, G. B., and Brinckerhoff, W. R., I. The pathological anatomy and histology of variola, *J. Med. Research*, 1904, xi, 12.

Ewing, J., The structure of vaccine bodies in isolated cells, *J. Med. Research*, 1904–05, xiii, 233.

Gins, H. A., Untersuchungen über die für Variola und Vaccine spezifischen Zellveränderungen, *Z. Hyg. u. Infectionskrankh.*, 1922, xcv, 255.

Harde, E. S., Some observations on the virus of vaccinia, in Mémoires publiés à l'occasion du Jubilé de Élie Metchnikoff (16 Mai 1915), Paris, 1921, xii, 107.

Hogue, M. J., A comparison of an amœba, *Vahlkampfia patuxent*, with tissue culture cells, *J. Exp. Zool.*, 1922, xxxv, 1.

Jungmann, P., Das wolhynische Fieber, Berlin, 1919.

Lipschütz, B., Die ätiologische Erforschung der "Einschlusskrankheiten" der Haut, *Zentr. Haut- u. Geschlechtskrankh.*, 1921–22, iii, 3.

MacCallum, W. G., and Oppenheimer, E. H., Differential centrifugalization: A method for the study of filterable viruses, as applied to vaccinia, *J. Am. Med. Assn.*, 1922, lxxviii, 410.

Mallory, F. B., Scarlet fever. Protozoon-like bodies found in four cases, *J. Med. Research*, 1903–04, x, 483.

Paschen, E., Über den Erreger der Variolavaccine. Immunitätsverhältnisse bei Variolavaccine, in Kraus, R., and Levaditi, C., Handbuch der Technik und Methodik der Immunitätsforschung, Jena, 1911, suppl. 1, 465.

von Prowazek, S., Chlamydozoa. I. Zusammenfassende übersicht, *Arch. Protistenk.*, 1907, x, 336.

von Prowazek, S., Vaccine, in von Prowazek, S., Handbuch der pathogenen Protozoen, Leipsic, 1912, i, 122.

von Prowazek, S., and Lipschütz, B., Chlamydozoen. Allgemeines, in von Prowazek, S., Handbuch der pathogenen Protozoen, Leipsic, 1912, i, 119.

da Rocha-Lima, H., Beobachtungen bei Flecktyphusläusen, *Arch. Schiffs- u. Tropen-Hyg.*, 1916, xx, 17.

Ségal, J., The association of the virus of typhus fever with the various blood elements, *Brit. J. Exp. Path.*, 1922, iii, 95.

Simpson, M. E., Vital staining of human and mammalian blood, with special reference to the separation of the monocytes, *Anat. Rec.*, 1922, xxiii, 37.

Solovief, P., Examen histologique de quelques pièces de trachome expérimental du lapin et du magot, *Arch. Inst. Pasteur*, 1921, i, 388.

Steinhardt, E., Israeli, C., and Lambert, R. A., Studies on the cultivation of the virus of vaccinia, *J. Infect. Dis.*, 1913, xiii, 294.

Steinhardt, E., and Lambert, R. A., Studies on the cultivation of the virus of vaccinia. II, *J. Infect. Dis.*, 1914, xiv, 87.

Wolbach, S. B., Todd, J. L., and Palfrey, F. W., The etiology and pathology of typhus, Cambridge, 1922.

Woodcock, H. M., An introduction to the study of hæmatophagy, *J. Roy. Army Med. Corps*, 1921, xxxvii, 418.

EXPLANATION OF PLATES.

PLATE 58.

The figures have been drawn with a Zeiss apochromatic objective of 1.5 mm., compensating ocular 6, and camera lucida at the table level, giving a magnification of 1,500 diameters. All represent living corneal cells in physiological saline solution viewed by direct illumination. Figs. 1, 18, and 19 are unstained cells. Fig. 20 is a cell supravitally stained with Janus green B and the remainder are of cells stained with brilliant cresyl blue 2 B. The corpuscles of Guarnieri, or vaccine bodies, were colored pink and blue, while the nuclei remained quite unstained.

FIG. 1. Living, unvaccinated, and unstained corneal cell in which rod-like mitochondria and other granular material may be distinguished.

FIG. 2. Cell from the same animal, supravitally stained with brilliant cresyl blue, showing traces of the material of which the vaccine bodies are built.

FIG. 3. Cell 4 days after vaccination, supravitally stained in the same way, showing a larger amount of the same material.

FIG. 4. Cell 5 days after vaccination similarly treated. The material shows signs of heterogeneity in the form of clear unstained vacuoles.

FIG. 5. Cell 4 days after vaccination, similarly stained, showing the spread along the surface of the nucleus of a vaccine body, in which it is possible to distinguish materials of different staining reaction.

FIG. 6. Cell from the same cornea, stained in the same way, containing a larger vaccine body, exhibiting both unstained vacuoles and blue-stained granules.

FIG. 7. Cell 2 days after vaccination similarly stained.

FIG. 8. Cell 5 days after vaccination, stained in the same way and containing a heavy deposit of material.

FIG. 9. Cell 2 days after vaccination stained in the same way.

FIG. 10. Cell 1 day after vaccination, containing several small heterogeneous vaccine bodies.

FIG. 11. Cell 5 days after vaccination, with a very heavy deposit of material.

FIG. 12. Cell 5 days after vaccination, with a large solitary vaccine body.

FIG. 13. Cell 2 days after vaccination, containing a solitary vaccine body in which large and small droplets may be distinguished, which took the blue stain.

FIG. 14. Cell 5 days after vaccination, containing several vaccine bodies with droplets (or granules) which took a blue coloration.

FIG. 15. Cell 5 days after vaccination, containing a very large solitary and apparently homogeneous vaccine body.

FIG. 16. One corneal cell phagocytosed by another in a 5 day lesion.

FIG. 17. Cell from the same cornea, containing a vaccine body in two parts staining differently.

FIG. 18. Unstained corneal cell 7 days after vaccination, containing two large and slightly refractile vaccine bodies closely applied to the nuclear membrane. The finer granulations may be resolved into indistinct rod-like mitochondria and a few highly refractile droplets of lipoid-like material.

FIG. 19. Corneal cell from the same lesion, also unstained. It contains a mass of coccal and bacillary bodies which is only very occasionally met with.

FIG. 20. Cell from a 7 day vaccination, supravitally stained with Janus green to demonstrate more sharply the mitochondria. It contains a vaccine body as large as the clear unstained nucleus.

PLATE 59.

These figures have been drawn under the same magnification. All of them represent fixed and stained preparations. At least two factors are concerned in their small size, as compared with Figs. 1 to 20: first, the shrinkage resulting from the technique employed, and second, the tendency of the living cells illustrated in Figs. 1 to 20 to become slightly flattened, thus increasing their apparent size, but not their actual volume.

FIG. 21. Normal corneal cell from a control animal fixed in Zenker's fluid and colored with Giemsa's stain. The irregular deposits of material in the cytoplasm are colored dark blue in the original preparation. On infection this gives rise to typical vaccine bodies.

FIG. 22. Cell about 2 mm. from the lesion in a cornea 1 day after vaccination, fixed in Zenker's fluid without acetic acid and colored with Giemsa's stain. The same irregular material is again colored dark blue.

FIGS. 23 and 24. Cells 5 days after vaccination, fixed and stained in the same way, showing an increase in the material giving rise to vaccine bodies.

FIG. 25. Cell 6 days after vaccination, fixed and stained in the same way, representing very high development of the small bodies.

FIG. 26. Cell 5 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, illustrating the formation of larger vaccine bodies.

FIG. 27. Cell from a lesion of 6 days, fixed in Zenker's fluid and colored with Wright's stain, containing a large vaccine body stained light blue (robin's egg color), partly surrounded by a mantle of dark blue material, and other vaccine bodies disposed just beneath the cell membrane.

FIG. 28. Cell 4 days after vaccination, fixed in Zenker's fluid and stained with carbolfuchsin and acid violet which shows up the vaccine bodies in crimson against a light green background. It indicates an accommodation in the shape and position of the vaccine bodies to that of the cell.

FIG. 29. Cell from a 4 day lesion, fixed in acetic-sublimate and colored with Giemsa's stain, containing a very deeply colored and homogeneous nucleus.

FIG. 30. Cell from a 5 day lesion, fixed in Zenker's fluid and colored with Giemsa's stain, showing a large vaccine body containing clear droplets of uncolored material, closely applied to the nuclear membrane.

FIG. 31. Cell 1 day after vaccination, fixed in Zenker's fluid without acetic acid and colored with Giemsa's stain, containing a solitary spherical vaccine body.

FIG. 32. Cell from the same preparation possessing a smaller single vaccine body.

FIG. 33. Cell 6 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, showing the hour-glass-like indentation of the nucleus by the vaccine bodies.

FIG. 34. Cell 6 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, showing vaccine bodies occupying depressions in the nuclear membrane.

FIG. 35. Cell 2 days after vaccination, fixed in Giemsa's sublimate mixture and colored with Giemsa's stain, showing solitary and accessory vaccine bodies.

FIG. 36. Cell from a lesion of 6 days, fixed in Zenker's fluid and colored with Wright's stain, containing a large vaccine body stained light blue (robin's egg tint), with an irregular envelope of very dark purplish blue material.

FIG. 37. Cell 5 days after vaccination, representing the pocket-like indentation of the nucleus.

FIG. 38. Cell from the same preparation as Fig. 34.

FIG. 39. One corneal cell phagocytosed by another containing well developed vaccine bodies, 5 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain.

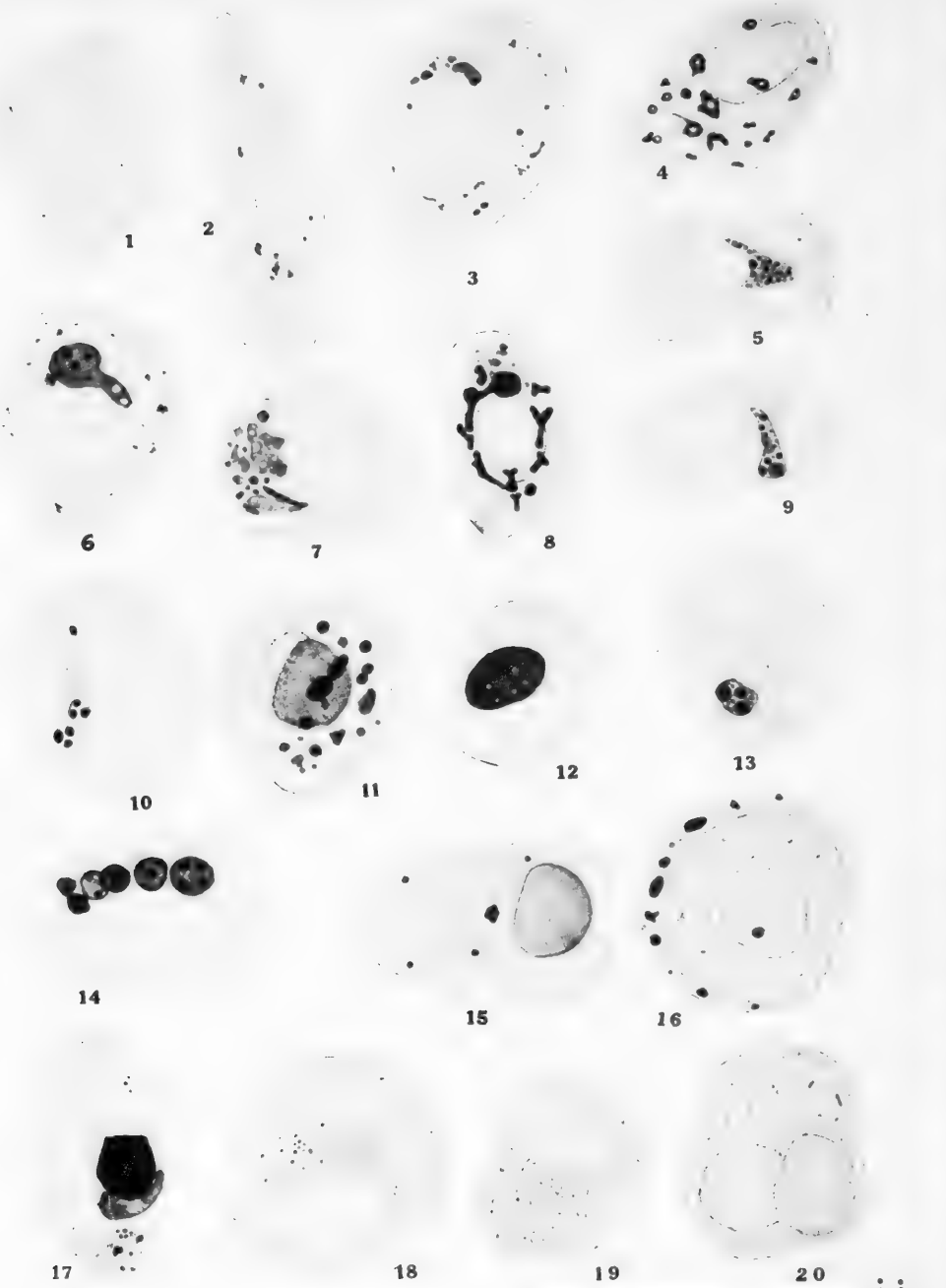
FIG. 40. A similar act in phagocytosis, in which both cells contain vaccine bodies, from a cornea 6 days after vaccination, fixed in Zenker's fluid, stained with methyl violet, treated with Gram's solution, and decolorized in 95 per cent alcohol. The vaccine bodies retain the stain much more strongly than do the nuclei. One of the bodies in the phagocytosed cell is made up of both Gram-positive and Gram-negative material.

FIG. 41. Cell from a cornea 5 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, containing a large vaccine body, the center of which was colored dark blue and the periphery light blue.

FIG. 42. Cell from the same preparation as Fig. 35, illustrating also nuclear infoldings and halos.

FIGS. 43 and 44. Constricted and binucleated cells from the same specimen as Fig. 41.

FIG. 45. Cell distant about 4 mm. from the lesion in a cornea 3 days after vaccination, fixed in Altmann's fluid and stained with fuchsin and methyl green. The mitochondria are rod-like and filamentous. They stained bright red in sharp contrast to the homogeneous green-colored cytoplasm in which the material destined to form the vaccine bodies is not visible.



(Cowdry: Supravital staining of vaccine bodies.)

(Cowdry: Supravital staining of vaccine bodies.)

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

X. The Immunizing Effects in Rabbits of Subcutaneous Injections of Killed Cultures of Bacterium pneumosintes.

By PETER K. OLITSKY, M.D., and FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 18, 1922.)

It is evident from the results of the serological experiments described in Papers VII[1] and IX[2] of these studies that the reaction of the animal body to intratracheal and intravenous injections of living *Bacterium pneumosintes* derived from the nasopharyngeal secretions of influenza patients involves the production of serum antibodies. Evidence of the protective effects of such injections against subsequent infection with *Bacterium pneumosintes* has also been reported.[2,3]

It appears, therefore, that *Bacterium pneumosintes* has the antigenic character common to bacteria in general, and that the mechanism of protection against it is similar to that which comes into play in the case of infections with other pathogenic microorganisms. If this is so, it follows that subcutaneous injections of killed cultures of *Bacterium pneumosintes* may be expected to induce a similar protective reaction. Recently, we have had the opportunity to put this hypothesis to experimental test. A study of the serological and immunological reactions of rabbits to prophylactic subcutaneous injections of killed *Bacterium pneumosintes* is reported in the present paper.

The opportunity for such a study was afforded by the recurrence of epidemic influenza in New York City in the early months of 1922. This recurrence provided us with four new strains of *Bacterium*

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 553.
[2] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxvi, 501.
[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 1.

pneumosintes which had not undergone prolonged artificial cultivation and were, therefore, pathogenic for rabbits.[2] With these the protective effects of subcutaneous injections could be tested, both against subsequent infection of the lungs with *Bacterium pneumosintes*, and, as a corollary, against the secondary invasion of the lungs by other bacteria. The study also furnished evidence of the local and general reactions which might be expected to follow the subcutaneous injection of killed cultures of *Bacterium pneumosintes*, and thus prepared the way for similar observations in man.

Methods.

Preparation of the Killed Cultures of Bacterium pneumosintes.—The experimental evidence [1,2,3] points to the antigenic identity of all of our strains of *B. pneumosintes*, whether derived from the epidemic of 1918–19, the recurrence of 1920, or the recent recurrence of 1922. It did not seem advisable, therefore, to prepare a polyvalent vaccine of all the available strains, but, in order to include possible antigenic differences, which the serological tests did not disclose, two cultures were chosen and used in equal quantities,—Strain 17, isolated in 1918 directly from the nasopharyngeal secretions of an influenza patient, and Strain 34, obtained in February, 1922, from lung tissue derived from a rabbit in the second passage of the active agent from Case 34.

The organisms were grown for 3 to 5 days in 100 cc. Florence flasks of *B. coli* broth,[2] seeded with 1 cc. of a dextrose broth-rabbit kidney culture of Strain 17 or 34. After centrifugation the supernatant medium was decanted and the sediment suspended in 0.85 per cent salt solution and heated for 30 minutes at 56–60°C. The suspension was then standardized on an opacity scale and tested for sterility.

Standardization of the Suspensions.—Because of wide differences in the size of bacteria, the standardization of suspensions by counting methods does not afford comparable data on the amount of bacterial substance present, especially since the accepted error in counting methods is often as great as 25 per cent. On the other hand, it is convenient to speak of bacterial suspensions in terms of the bacterial count. What is lacking is a comparative standard to which bacteria of various sizes can be referred.

We have adopted *Staphylococcus albus* as a convenient standard and have correlated the bacterial count and the opacity of its suspensions in a number of tests. Using the Wright method for counting and the depth of disappearance readings described by one of us (Gates[4]), it was found that 1,000 million heat-killed staphylococci corresponded to a corrected opacity of 3.47 cm.

---

[4] Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

Regardless of the actual number of *B. pneumosintes* in a given suspension, we have standardized the killed cultures on the staphylococcus scale. For example, a suspension of *B. pneumosintes* which has a corrected opacity of 3.47 cm. is said to contain $\frac{1{,}000 \text{ million}}{\text{S. E.}}$ organisms (Staphylococcus Equivalent). In other words, the amount of bacterial substance present is assumed to correspond approximately to the amount contained in a suspension of 1,000 million staphylococci per cc. Suspensions of *B. pneumosintes*, standardized by this method, have been checked by comparison with equivalent quantities of *Staphylococcus albus*, *B. typhosus*, and Meningococcus Type A in Hopkins tubes.[5] The amounts of bacterial sediment obtained corresponded within the limit of observation of the tubes.

Procedure.—Twenty-six apparently healthy adult rabbits were given three subcutaneous injections of heat-killed *Bacterium pneumosintes*, at intervals of 5 days. With the Staphylococcus Equivalent as a measure, the doses chosen were $\frac{2{,}000 \text{ million}}{\text{S. E.}}$, $\frac{4{,}000 \text{ million}}{\text{S. E.}}$, and $\frac{4{,}000 \text{ million}}{\text{S. E.}}$ *Bacterium pneumosintes*, a total for each animal of $\frac{10{,}000 \text{ million}}{\text{S. E.}}$. The injected suspensions contained equal quantities of Strains 17 and 34, in 1 to 2 cc. of physiological saline solution. In order to localize the subcutaneous reaction, the injections were made through a shaved area, cleansed with soap and water and alcohol, on the most dependent portion of the abdomen.

Reactions.—None of the rabbits showed any significant local reactions or rise in temperature following the first injection of $\frac{2{,}000 \text{ million}}{\text{S. E.}}$ organisms. After the second injection $\left(\frac{4{,}000 \text{ million}}{\text{S. E.}}\right)$ most of the animals showed a mild, local reaction, consisting of redness, heat, and swelling which persisted for 2 days. The third injection rarely caused local symptoms. No general symptoms referable to the inoculations were noted. One rabbit was found dead on the 2nd day after the third injection, and two others were found to be suffering from an infection prevalent among the stock rabbits, and were killed.

[5] Hopkins, J. G., *J. Am. Med. Assn.*, 1913, lx, 1615.

Four other rabbits were given the total dose of $\frac{10{,}000 \text{ million}}{\text{S. E.}}$ killed culture of *Bacterium pneumosintes* in a single subcutaneous injection. Two of these rabbits developed localized areas of heat, redness, and swelling. The other two showed practically no local reaction. Blood counts made on these animals before and after injection showed no immediate change in the total leucocyte count, or in the proportion of mononuclear cells, such as is characteristic of infection with the living organisms. The body temperatures were not affected by the injection.

Agglutinin Formation.

From 10 to 32 days after the third subcutaneous injection, nineteen of the rabbits were used in the protection experiments to be described. The blood serum of fifteen of them was tested for the presence of specific agglutinins, 10 to 27 days after the final injection. Of these sera, four failed to agglutinate the homologous strains of *Bacterium pneumosintes* in the lowest dilution tested, 1:4. Five sera, in this dilution, showed agglutinins against one or both strains. Five other sera agglutinated the strains when diluted 1:8, and one in a dilution of 1:16. In view of the individual variations in response among rabbits, and the relatively low agglutinogenic properties of *Bacterium pneumosintes*[2] these results are what would be expected. They indicate that the subcutaneous injection of killed cultures of *Bacterium pneumosintes* induces antibody formation. But the critical test of immunization is not the titer of serologically demonstrable antibodies, but protection against subsequent infection.

As a preliminary to the series of protection experiments with the subcutaneously inoculated animals, the four rabbits that were injected with a single dose of $\frac{10{,}000 \text{ million}}{\text{S. E.}}$ organisms were tested for lowered resistance on the day following inoculation. Attention has repeatedly been called to one of the characteristic features of infection with *Bacterium pneumosintes;* namely, the loss of resistance to secondary infection which occurs in the injured lung.[6,7] In

[6] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 373.
[7] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiv, 1.

view of the nature of the lesions caused by *Bacterium pneumosintes* in the pulmonary tissues, and the localization in these damaged tissues of other bacteria, injected intravenously, it is natural to suppose that it is the local disorganization and destruction of the lung structure that opens the way to secondary infections. Nevertheless, it was conceivable that *Bacterium pneumosintes* might have some inherent, specific property of promoting infections by other organisms and it seemed important to know whether, in the absence of a pulmonary lesion, the presence of *pneumosintes* protein in the body might reduce the natural resistance to other bacteria during what is commonly called a negative phase.

To test this possibility, two of the four rabbits that received $\frac{10{,}000 \text{ million}}{\text{S. E.}}$ killed organisms in one dose were injected intravenously on the following day with a dose of atypical Type II pneumococcus which was harmless to normal animals but infective for those suffering from a pulmonary lesion induced by *Bacterium pneumosintes*.

One of the animals remained well, gave negative blood cultures on the 2nd day, and, when killed for autopsy on that day, showed no lesions of the lungs, cultures of which remained sterile. The other rabbit died on the day following injection. No obvious lesions were found. The blood culture, post mortem, yielded the bacillus of rabbit septicemia. This infection, from which two other vaccinated rabbits were found to be suffering, was prevalent among the stock. Since the subcutaneous injection of killed cultures of *Bacterium pneumosintes* apparently did not lower the resistance of the two animals to the pneumococcus, these accidental infections presumably bear no relation to the problem in hand.

The third rabbit was similarly injected with a non-infective dose of *Streptococcus hæmolyticus*, and the fourth with *Bacillus pfeifferi*. Neither streptococcus nor Pfeiffer's bacillus could subsequently be recovered by blood culture, and at autopsy the organs of the rabbits, including the lungs, appeared normal. Cultures of the lung tissue were negative.

Protection Experiments.

Experiments to test the protective efficacy of the subcutaneous injections of killed cultures were divided into three groups.

The vaccinated rabbits in Group A were injected intratracheally with the active agent of rabbit passages in the 1922 series, in doses that proved infective for normal control animals. The vaccinated rabbits in Group B were intratracheally injected with mass cultures of pathogenic (1922) strains of *Bacterium pneumosintes*. The vaccinated rabbits in Group C were injected intratracheally with *Bacterium pneumosintes*, and then intravenously with other bacteria, in doses which, following a similar intratracheal injection of *Bacterium pneumosintes*, caused pneumonia in uninoculated controls. Typical protocols may be cited to illustrate each group.

Group A. To Test the Resistance of Vaccinated Rabbits to Pathogenic Doses of the Active Agent of Rabbit Passages. Experiment 1.—Rabbit A (vaccinated): Last subcutaneous injection Apr. 29, 1922. May 9. Leucocytes 5,000, of which 2,200 were mononuclear cells. Temperature 39.3°C. May 10. Injected intratracheally with 3 cc. of a salt solution suspension of ground lung tissue derived from a rabbit in the third passage of the active agent from a recent case of early epidemic influenza (Case 28[2]). May 11. Leucocytes 5,200, mononuclears 3,224. Temperature 39.3°C. May 12. Leucocytes 9,000, mononuclears 5,670. Killed. Lungs and other organs show no lesions. Aerobic and anaerobic cultures of lung tissue yielded no growth.

Rabbit B (normal control): May 9. Leucocytes 14,400, of which 6,336 were mononuclear cells. Temperature 39.3°C. Conjunctivæ normal. May 10. Intratracheally injected with 3 cc. of the ground lung suspension given to Rabbit A. May 11. Leucocytes 11,000, mononuclears 4,840. Temperature 39.4°C. Conjunctivitis. May 12. Leucocytes 8,400, mononuclears 2,856. Temperature 39.5°C. Conjunctivitis. Killed. Only the lungs showed lesions. Both lungs were the site of a hemorrhagic edema and an emphysema, without consolidation. Microscopically the affected tissues showed marked edema of the interalveolar strands with a cellular exudate of mononuclear and polymorphonuclear cells. The lumina of the bronchi were filled with serum, leucocytes, and cells from the necrotic and exfoliated epithelium. Among the cilia of the bronchial epithelium clumps of minute bacteria, morphologically similar to *B. pneumosintes*, were noted. Anaerobic, as well as aerobic cultures, failed to yield a growth, however. A repetition of this experiment gave the same results.

The experiments illustrated by these typical instances show that subcutaneous injections of killed cultures of *Bacterium pneumosintes* cause the development of an effective resistance to pathogenic doses of the active agent derived from the nasopharyngeal secretions of influenza patients. The identity of this active agent with *Bacterium*

pneumosintes has already been demonstrated. Tissue suspensions were used because it was thought that *Bacterium pneumosintes* contained in the lungs of affected rabbits after repeated passages might be enhanced in virulence and pathogenicity, and hence afford a more severe test of resistance than was to be obtained with the laboratory strains which were studied in Group B.

Group B. To Test the Resistance of Vaccinated Rabbits to Pathogenic Doses of B. pneumosintes. Experiment 2.—Rabbit A (vaccinated): Last subcutaneous injection Apr. 29, 1922. May 15. Leucocytes 7,200, of which 2,880 were mononuclear cells. Temperature 39.2°C. Conjunctivæ normal. Injected intratracheally with 3 cc. of a salt solution suspension of *B. pneumosintes*, Strain 34, representing the third generation in mass culture. May 16. Leucocytes 8,000, mononuclears 4,160. Temperature 39.2°C. Conjunctivæ normal. May 17. Leucocytes 7,200, mononuclears 4,176. No change in temperature. Conjunctivæ normal. Killed. No lesions found in the lungs or other organs. Aerobic and anaerobic cultures of the lung tissue remained sterile.

Rabbit B (normal control): May 15. Leucocytes 12,000, of which 5,760 were mononuclear cells. Temperature 38.5°C. Conjunctivæ normal. Injected precisely as was Rabbit A. May 16. Leucocytes 10,000, mononuclears 4,400. Temperature 39.5°C. Conjunctivitis. May 17. Leucocytes 6,000, mononuclears 2,700. Temperature 39.6°C. Killed. The lungs showed the hemorrhagic edema, emphysema, cellular reaction, and bronchial lesions already described as characteristic of the effects of *B. pneumosintes*.[8] Among the cilia of the bronchial epithelium and in the interalveolar tissues, numerous clumps of minute microorganisms, morphologically identical with *B. pneumosintes*, were found. Aerobic cultures of the lung tissue showed no growth; anaerobic cultures yielded *B. pneumosintes*.

This experiment is typical of the five in Group B. In spite of the fact that a large dose of a pathogenic strain of *Bacterium pneumosintes* was employed, the protection afforded by the previous subcutaneous injections of killed culture was sufficient to prevent invasion of the lung.

Since the intratracheal injections of *Bacterium pneumosintes* failed to infect and to produce pathological lesions in the lungs of the vaccinated rabbits in Groups A and B, these animals presumably would have shown a normal resistance to pulmonary infection with other bacteria. The rôle of secondary infections in human influenza is so

[8] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

important, however, and the reduction of pulmonary resistance induced by *Bacterium pneumosintes* in experimental animals is so characteristic and significant, that we were led to test the resistance of vaccinated rabbits to other bacteria, following intratracheal injections of *pneumosintes* cultures. These experiments comprise Group C of the present series and were carried out with an atypical Type II pneumococcus, a hemolytic streptococcus, and *Bacillus pfeifferi.*

In this group of experiments the procedure described in Paper V of this series[7] was employed. First, cultures of atypical Type II pneumococcus, *Streptococcus hæmolyticus*, and *Bacillus pfeifferi* were injected intravenously into normal rabbits and a non-infective dose was determined in each case. Then the pathogenicity of mass cultures of the 1922 strains of *Bacterium pneumosintes*, injected intratracheally into normal rabbits, was confirmed (Control Rabbit B, Group B). Finally the animals previously prepared by vaccination, and normal controls, were injected intratracheally with the tested mass cultures of *Bacterium pneumosintes*, and on the following day each was injected intravenously with the dose of one of the organisms above mentioned that had proved non-infective for normal rabbits. The results of these experiments are illustrated by the following protocols.

Group C. To Test the Resistance of Vaccinated Rabbits to Infection with Other Bacteria Following the Intratracheal Injection of B. pneumosintes. Experiment 3. B. pneumosintes and the Pneumococcus.—Rabbit A (vaccinated): Last injection May 2, 1922. May 16. Leucocytes 9,300, of which 2,140 were mononuclears. Temperature 39.8°C. Injected intratracheally with 3 cc. of a mass culture of *B. pneumosintes* similar to that used in Experiment 2. May 17. Leucocytes 11,400, mononuclears 4,450. Temperature 39.9°C. Injected intravenously with 3 million atypical Type II pneumococci, suspended in 1 cc. of salin. solution. (This was the tested non-infective dose for this culture of pneumoe coccus.) May 18. Leucocytes 7,600, mononuclears 3,570. Temperature 40.1°C. May 19. Leucocytes 12,000, mononuclears 5,760. Temperature 40.2°C. May 20. Leucocytes 8,600, mononuclears 4,730. Temperature 40°C. Blood taken for culture yielded no growth. Killed. Lungs and other organs appeared normal. Aerobic and anaerobic cultures of the lung tissue remained sterile.

Rabbit B (normal control): May 16. Leucocytes 6,300, of which 3,090 were mononuclears. Temperature 39.2°C. Conjunctivæ normal. Injected intratracheally with *B. pneumosintes*, as was Rabbit A. May 17. Leucocytes 6,000, mononuclears 1,200. Temperature 39.5°C. Conjunctivitis. Injected intra-

venously with 3 million pneumococci, as was Rabbit A. May 18. Leucocytes 5,600, mononuclears 2,520. Temperature 41.2°C. Conjunctivitis. May 19. Leucocytes 18,800, mononuclears 7,520, polymorphonuclears 11,280. Temperature 40.7°C. Conjunctivitis. Blood culture made on this day yielded the pneumococcus. Killed. Lungs voluminous with edema and emphysema and showed numerous hemorrhages. Lower right lobe dark, solid, and resistant to section. Fibropurulent discharge from bronchi. Microscopically, hemorrhage, edema, and emphysema were noted in some areas. Other areas were the site of a peribronchial consolidation and polymorphonuclear exudate. Impression smears of the cut lung showed numerous polymorphonuclear cells, and free and phagocytosed pneumococci. Aerobic and anaerobic lung cultures yielded pneumococci. Diagnosis: pneumococcus septicemia and beginning pneumonic consolidation.

This experiment is one of three in which it was found that the pneumococcus, injected intravenously, following an intratracheal injection of *Bacterium pneumosintes*, produced a septicemia and pneumococcus pneumonia in control rabbits but not in rabbits previously vaccinated with killed cultures of the primary organism. One experiment was complicated by the presence of *Bacillus lepisepticus* in the lung tissues, but not in the blood of the vaccinated animal. The lungs of this rabbit showed no pneumonic consolidation, and cultures of lung and blood were negative for the pneumococcus.

Experiment 4. B. pneumosintes and Streptococcus hæmolyticus.—Rabbit A (vaccinated): Last injection May 2, 1922. May 22. Leucocytes 5,800, of which 3,070 were mononuclears. Temperature 39.7°C. Conjunctivæ normal. Injected intratracheally with 3 cc. of a saline solution suspension of a mass culture of *B. pneumosintes*, similar to that used in Experiments 2 and 3. May 23. Leucocytes 6,800, mononuclears 3,400. Temperature 39.3°C. Conjunctivæ normal. Injected intravenously with a saline solution suspension of *Streptococcus hæmolyticus* containing 1 million microorganisms (the tested non-infective dose for this culture). May 24. Leucocytes 8,400, mononuclears 5,376. Temperature 39.2°C. Conjunctivæ normal. May 25. Leucocytes 6,600, mononuclears 3,498. Temperature 39.2°C. Conjunctivæ normal. Blood cultures taken on this day remained sterile. May 26. Leucocytes 6,600, mononuclears 3,894. Temperature 39.4°C. Conjunctivæ normal. Blood culture free from growth. Killed. Lungs and other organs appeared normal. Aerobic and anaerobic cultures of the lung tissue yielded no growth.

Rabbit B (normal control): May 22. Leucocytes 7,600, of which 2,812 were mononuclears. Temperature 38.4°C. Injected intratracheally with *B. pneumosintes*, precisely as was Rabbit A. May 23. Leucocytes 7,000, mononuclears 1,680. Temperature 39.5°C. Conjunctivitis. Injected intravenously with

1 million *Streptococcus hæmolyticus*. May 24. Leucocytes 8,000, mononuclears 2,240. Temperature 38.9°C. Conjunctivitis. May 25. Leucocytes 5,400, mononuclears 3,240. Temperature 39°C. Conjunctivæ normal. Blood culture positive for *Streptococcus hæmolyticus*. May 26. Leucocytes 6,800, mononuclears 1,700. Temperature 39.4°C. Conjunctivæ normal. Blood culture yielded *Streptococcus hæmolyticus*. Killed. Lungs showed diffuse consolidation of contiguous parts of left upper and lower lobes (red hepatization). Remainder of lung voluminous, edematous, and emphysematous, and showed a number of small hemorrhages on surface and on section. An impression smear showed numerous polymorphonuclear cells and a few streptococci. Cultures of the lung yielded *Streptococcus hæmolyticus*. Diagnosis: streptococcus septicemia and pneumonia.

Two other similar experiments with *Bacterium pneumosintes* and *Streptococcus hæmolyticus* gave similar results. As in the experiments with the pneumococcus, the previous vaccination of the experimental rabbits with killed cultures of *Bacterium pneumosintes* protected the animals against pathogenic doses of the living organisms; and in the absence of a pulmonary lesion, due to *Bacterium pneumosintes*, they maintained their normal resistance to secondary infection with the streptococcus.

Experiment 5. B. pneumosintes and B. pfeifferi.—Rabbit A (vaccinated): Last injection May 27, 1922. June 6. Leucocytes 9,000, of which 4,320 were mononuclears. Temperature 39.7°C. Injected intratracheally with 3 cc. of a saline solution suspension of a mass culture of *B. pneumosintes*. June 7. Leucocytes 11,600, mononuclears 4,872. Temperature 39.5°C. Conjunctivæ normal. Injected intravenously with 7.5 billion *B. pfeifferi* (the tested non-infective dose of this culture) in 1 cc. of saline solution. June 8. Leucocytes 12,800, mononuclears 6,400. Temperature 39.5°C. Conjunctivæ normal. Blood culture remained sterile. June 9. Leucocytes 9,000, mononuclears 5,040. Temperature unchanged. Conjunctivæ normal. Blood cultures yielded no growth. Killed. The lungs and other organs appeared normal. Aerobic and anaerobic cultures showed no growth.

Rabbit B (normal control): June 6. Leucocytes 14,000, of which 7,420 were mononuclears. Temperature 39.2°C. Conjunctivæ normal. Injected intratracheally with *B. pneumosintes*, precisely as was Rabbit A. June 7. Leucocytes 12,800, mononuclears 3,200. Temperature 39.6°C. Conjunctivitis. Injected intravenously with 7.5 billion *B. pfeifferi*. June 8. Leucocytes 44,000, of which 5,300 were mononuclears and 38,700 were polymorphonuclear cells. Temperature 39.9°C. Conjunctivitis. Blood culture yielded *B. pfeifferi* in pure culture. June 9. Leucocytes 22,800, mononuclears 4,788, polymorphonuclears 18,012. Temperature 39.9°C. Conjunctivitis. Blood culture positive for *B. pfeifferi*.

Killed. Right lower lobe of lungs dark, congested, resistant to section, with a purulent exudate in the bronchi. Histological examination revealed edema, hemorrhages, emphysema, and peribronchial and lobular consolidation. The solid areas contained many polymorphonuclear cells, with some mononuclears and respiratory epithelium in a matrix of serum and fibrin. In some areas the bronchi were filled with leucocytes, serum, and fibrin, surrounded by a necrotic and exfoliated epithelium. Impression smears showed many polymorphonuclear cells and intra- and extracellular Gram-negative bacilli, resembling *B. pfeifferi*. Anaerobic cultures of the lung were negative, although minute organisms, resembling *B. pneumosintes*, were seen in the ciliary epithelium of affected bronchi. Diagnosis: septicemia and pneumonia, due to *B. pfeifferi*.

Six similar experiments were made in this series. The results of four of these corresponded to those illustrated above. In the other two experiments the subcutaneous injections failed to protect the animals, and the intratracheal injection of live *Bacterium pneumosintes*, followed by the intravenous injection of *Bacillus pfeifferi* induced a pulmonary infection to which the rabbits succumbed.

The failure of the vaccine to protect in these two instances may be attributed to individual susceptibility on the part of the rabbits, or possibly to the rather arbitrary dose of *Bacterium pneumosintes* employed. In each experiment the suspension of *Bacterium pneumosintes* for intratracheal injection was divided between the vaccinated and the control animals, 3 cc. to each, but in experiments with different cultures the potentialities of the dose employed may well have varied. Since the control animals in every instance were infected, it is certain that at least one infective dose was given in all, but in some experiments the rabbits may have received several multiples of this minimum. Thus the experiments may have been much more severe tests of the protection afforded by subcutaneous vaccination than the protocols indicate, and it was to be expected that in some instances protection would be incomplete.

SUMMARY AND CONCLUSIONS.

A series of rabbits was subcutaneously injected with three measured doses of killed cultures of two strains of *Bacterium pneumosintes* derived from the nasopharyngeal secretions of influenza patients. These rabbits were subsequently tested for the development of serum

antibodies and for the presence of an induced immunity to the living organisms, with the following results.

The serum of eleven of fifteen rabbits, tested from 10 to 27 days after the final subcutaneous injection, specifically agglutinated *Bacterium pneumosintes*, whereas normal rabbit serum did not.

Nineteen vaccinated rabbits were subjected to protection experiments. Two of them were unaffected by an intratracheal injection of *Bacterium pneumosintes*, contained in the lung tissues of previously infected animals, in a dose which typically affected the control rabbits. Fifteen of the other seventeen proved to be completely resistant when tested by intratracheal injections of *Bacterium pneumosintes* cultures that produced typical infections in the controls. Ten of these fifteen rabbits were injected intravenously with living cultures of pneumococcus, *Streptococcus hæmolyticus*, or *Bacillus pfeifferi* in doses which were non-infective under normal conditions, but infective, as experience has shown, in the presence of a primary lesion caused by *Bacterium pneumosintes*. In none of these animals did infection develop. The two remaining rabbits of the seventeen were not protected against *Bacterium pneumosintes* by the vaccination, and they further developed a secondary pulmonary infection with *Bacillus pfeifferi* after its intravenous injection. Control rabbits similarly injected intratracheally with *Bacterium pneumosintes*, and then intravenously with the pneumococcus, streptococcus, or *Bacillus pfeifferi* in doses that had proved non-infective for normal rabbits, uniformly developed a secondary infection with these organisms.

The mildness of the local reactions and the absence of general signs, following vaccination with *Bacterium pneumosintes*, indicate that similar injections would be well tolerated in man. There is no evidence that the subcutaneous injection of large doses of the heat-killed organisms reduces the resistance of the animal body to infections with other bacteria. In single rabbit experiments the resistance to intravenously injected pneumococci, streptococci, or *Bacillus pfeifferi* has been found unreduced immediately after vaccination with *Bacterium pneumosintes*.

STUDIES ON THE DISEASE OF GUINEA PIGS DUE TO BACILLUS ABORTUS.

By WILLIAM A. HAGAN, D.V.M.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, July 7, 1922.)

HISTORY.

That a characteristic disease of guinea pigs could be produced by inoculation with cultures of *Bacillus abortus* was first pointed out by Smith and Fabyan (1912). A more detailed study of the disease was made by Fabyan (1912). Since that time the disease has become well known to workers in animal pathology, a great many of whom have utilized the susceptibility of the guinea pig to infection with this organism as a means of detecting it in animal tissues and discharges.

The observations on this disease which are recorded in this paper are largely on the pathogenicity of the organism and the resistance of the animal to infection. The data were collected preliminary to, and in connection with an experiment to determine the value of heat-killed cultures of the organism in producing an active immunity to *Bacillus abortus*, the results of which are reported in one of the accompanying papers (Hagan, 1922).

The Minimum Infective Dose.

Since it is a well recognized fact that most immunities are but relative and may be overwhelmed by a large amount of infection, though perhaps functioning perfectly for a reasonable amount, an attempt was made, in connection with the immunization experiment just mentioned, to determine the minimum amount of culture needed to produce infection regularly.

Search of the literature failed to show any data on this point. Smith and Fabyan had used comparatively large amounts of material in their work. Smil-

lie (1918) had found, in one experiment, that a standard suspension, which he had been using in his work, could be diluted at least 64 times without altering appreciably the disease process in his guinea pigs. Further than this he had not gone, but it was evident that he had not nearly approached the minimum.

The strain of *B. abortus* used in this work was isolated from the placenta of a cow (No. 898) by passage through a guinea pig. The culture had subsequently been passed through a second guinea pig immediately before use. A plain veal infusion agar slant was inoculated with a culture from the spleen of the latter animal, the tube sealed, and incubated for 48 hours at 37°C. The resultant growth was washed from the surface of the slant with a small amount of physiological salt solution, making a uniform suspension. After a little shaking this was diluted with more salt solution to make a standard density which was determined by the method of Gates (1920). A reading of 2.4 cm. was selected arbitrarily as an end-point. Such a suspension is somewhat denser than a 24 hour typhoid culture in bouillon. Hereafter in this paper such a suspension will be referred to as a standard.

From this suspension, dilutions were made by transferring 1 cc. portions from one tube to the next in series, each tube containing 9 cc. of salt solution. Thus a series of dilutions was made as follows: 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, 1:1,000,000, 1:10,000,000, and 1:100,000,000. Intermediate dilutions of 1:5,000,000 and 1:50,000,000 were also made.

Preliminary work had shown that 1:100,000 dilutions of such suspensions were capable of infecting, and consequently higher dilutions were used in this series. Five guinea pigs, males, varying in weight from 355 to 410 gm. and of approximately the same age (Group V) were injected intraperitoneally with 1 cc. portions of the last five dilutions respectively. Infections resulted in the animals receiving dilutions of 1:1,000,000, 1:10,000,000, and 1:100,000,000; while the 1:5,000,000 and 1:50,000,000 failed to infect. Infection was determined by testing for the development of agglutinins and eventually, by killing the animals, observing lesions, and recovering *Bacillus abortus* culturally.

Infection by so minute a quantity of material as a 1:100,000,000 dilution of a standard suspension was a surprise. For confirmation, a second series of animals was tested with the same dilutions as before. Five male animals weighing from 415 to 470 gm. (Group V_2) received a 24 hour culture of the same strain as before. Just previous to use the strain had been recovered from another guinea pig at autopsy. In this case, infection of all animals occurred.

It was now evident that still higher dilutions of the suspension were necessary in order to approach the minimum infective dose. A third series of animals (Group V_3), consisting of eight animals weighing between 385 and 495 gm., was selected. The culture was of the same strain as before but had just been recovered from the heart's blood of a white mouse which had died within 24 hours after being inoculated with the strain. Before inoculation into the mouse the strain had been recovered from the spleen of a guinea pig which had been affected with a very severe form of the disease. The standard suspension was made up as before from a 24 hour culture, and from this in turn dilutions to 1:100,000,000, 1:200,000,000, 1:500,000,000, and 1:1,000,000,000. Two animals were injected with each of these dilutions. All became infected.

For the determination of the number of *Bacillus abortus* in the suspensions used for inoculation, the plating method described in one of the accompanying papers (Hagan, 1922) was used.

The degree of accuracy of this method is not known, as it was difficult to determine because of the small size of the organism. When several plates are made from one suspension, however, the discrepancy in the bacterial count of the various plates generally does not exceed 30 or 40 per cent, and frequently there is less than 10 per cent difference. It is advisable to add a drop of sterile blood to the surface of the agar plate at the time the suspension is added, so that the two will mix while spreading. The viscosity of the blood aids in making the film uniform over the surface, and somewhat higher counts result from the enrichment of the medium.

Although duplicate counts on a single suspension were reasonably consistent, it was found difficult to make up two suspensions which would give approximately the same count when using the Gates disappearing loop as a gauge. Thus four suspensions, standardized carefully with the Gates apparatus and plated in the same way, though at different times, gave the following counts: (*a*) 592, (*b*) 423, (*c*) 1,030, and (*d*) 1,451.

These figures represent the average counts of from two to four plates containing 0.1 cc. of a 1:1,000,000 dilution of the respective standards. The variation, amounting to over 300 per cent in the case of the extremes, must be due, in part at least, to inaccuracies in the method or operation of the opacity gauge by which the suspensions were standardized, although there is a large source of error in the pipetting when making up the dilutions. However, in estimating the number of bacteria injected into the guinea pigs, these discrepancies of count become of little consequence because of the great dilutions worked with.

If the counts given above may be accepted as representative of the true count of the living organisms in the suspensions, their average, which amounts to 874, should represent a mean which is within 100 per cent, one way or the other, of the true count in other suspensions made up in the same manner. In other words, a suspension of *B. abortus* made up in salt solution from the surface of a 24 hour slant agar culture, and having a reading of 2.4 cm. on the Gates gauge, has a mean calculated count of living organisms amounting to 8.74 billions per cc. with a minimum of about 4.5 billions and a maximum of about 13.0 billions. Since the guinea pigs were inoculated with dilutions running into the millions, these variations are reduced immediately to a few hundred organisms, at the most.

TABLE I.

Guinea pig No.	Dilution of standard suspension.	Computed No. of organisms injected.	Result.
	Group V.		
2059	1:1,000,000	8,740	+
2060	1:5,000,000	1,750	−
2058	1:10,000,000	874	+
2056	1:50,000,000	174	−
2055	1:100,000,000	87	+
	Group V_2.		
2172	1:1,000,000	8,740	+
2176	1:5,000,000	1,750	+
2179	1:10,000,000	874	+
2177	1:50,000,000	174	+
2175	1:100,000,000	87	+
	Group V_3.		
2232	1:100,000,000	87	+
2233	1:100,000,000	87	+
2230	1:200,000,000	44	+
2231	1:200,000,000	44	+
2228	1:500,000,000	18	+
2229	1:500,000,000	18	+
2227	1:1,000,000,000	9	+
2226	1:1,000,000,000	9	+

The minimum infective dose of *Bacillus abortus* for guinea pigs has not been determined, since infection was produced in the majority of the animals with the smallest amounts of material used. The computed number of bacteria used in the experiments is given in Table I.

Many workers who have been using guinea pig inoculations for diagnostic purposes have found it possible thus to detect, at times, *Bacillus abortus*, in material from which cultures had failed, the presumption being that sparsity of the organisms accounted for the differences in results. Grounds for believing in a rather high infectivity of the organism for the guinea pig have existed, therefore. This belief is amply substantiated by the data presented above. As a means of detecting *Bacillus abortus*, guinea pig inoculations are highly efficient.

Individual Variations in Susceptibility.

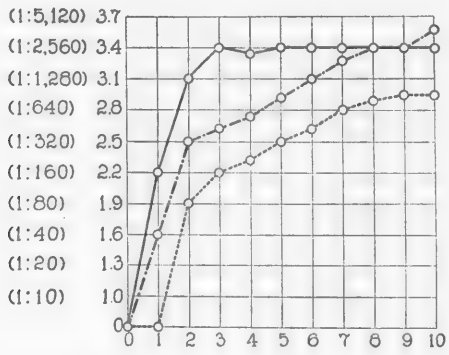
The failure of Guinea Pigs 2060 and 2056 (Table I, Group V) to become infected, although given from two to twenty times as much material as was needed to infect other animals, indicates a considerable degree of immunity in these animals. The resistance of No. 2056 was not tested subsequently, but No. 2060 was given a second inoculation at the time, and with the same material with which Group V_3 was infected (Table I). The dosage in this case was 1 cc. of a 1:1,000,000 dilution, and although this was from 100 to 1,000 times as great as that used in the animals of Group V_3, the response to the inoculation was much more tardy than that of any of the animals of the group mentioned. Although No. 2060 proved to be the most refractory of many animals tested, infection was accomplished by the second inoculation, which was of less than 10,000 organisms. Smillie reports two cases in which cultures were positive in certain parts of the alimentary tract of a bovine fetus, whereas the guinea pig inoculations from the same material gave negative results. While it is always possible that unequal distribution of the bacilli may produce such results, it is much more probable that resistant animals such as the two guinea pigs mentioned were used. The resistance would be effective only if very few organisms were present in the material injected.

The Effect of Variations in the Dosage on the Character of the Disease.

To determine the effects of dosage on the character of the resulting disease, a series of animals was inoculated, one-third with a heavy dose, another with a medium, and the other with a very small dose.

TABLE II.

Guinea pig No.	Inoculating dose.	Killed.	Spleen weight.	Spleen.	
				Organisms per gm.	Organisms per spleen.
		wks.	*gm.*		
2160	Undiluted.	1	2.18	269,700,000	587,946,000
2164	1:10,000	1	0.67	51,000	34,000
2165	1:1,000,000	1	0.63	450	280
2158	Undiluted.	4	1.02	2,600,000	2,650,000
2161	1:10,000	4	0.65	25,000	16,000
2166	1:1,000,000	4	0.69	106,000	73,000
2167	Undiluted.	6	1.39	24,000	34,000
2163	1:10,000	6	2.4	?	?
2157	1:1,000,000	6	1.67	39,000	65,000
2159	Undiluted.	9	3.02	48,000	145,000
2162	1:10,000	9	3.9	25,000	100,000
2168	1:1,000,000	9	1.13	1,400	2,500



TEXT-FIG. 1. Composite agglutinin curves of animals receiving different doses of *B. abortus*.

o———o Guinea Pigs 2158, 2160, 2167, and 2159. Standard suspension, undiluted.

o·—·—·—o Guinea Pigs 2161, 2163, 2164, and 2162. Dilutions of standard suspension, 1:10,000.

o·····o Guinea Pigs 2157, 2165, 2166, and 2168. Dilutions of standard suspension, 1:1,000,000.

Abscissæ represent weeks, ordinates logarithms of the dilutions. Blood dilutions are in parenthesis.

A standard suspension was used for the first, a 1:10,000 dilution of this for the second, and a 1:1,000,000 dilution for the third. Four guinea pigs were inoculated intraperitoneally with each of the suspensions. All animals were bled weekly and from each group one was killed at intervals of 1, 4, 6, and 9 weeks after the inoculation. The principal results are given in Table II and Text-fig. 1.

The principal effect of changing the amount of the infecting dose is to change the time relations. A very heavy inoculation calls forth early agglutinin production and early lesions, smaller doses produce nearly the same effect but at later periods; and the smaller the dose, the longer is this period. The factor of individual variations in resistance enters rather prominently into such data as those of Table II.

The Course of the Disease.

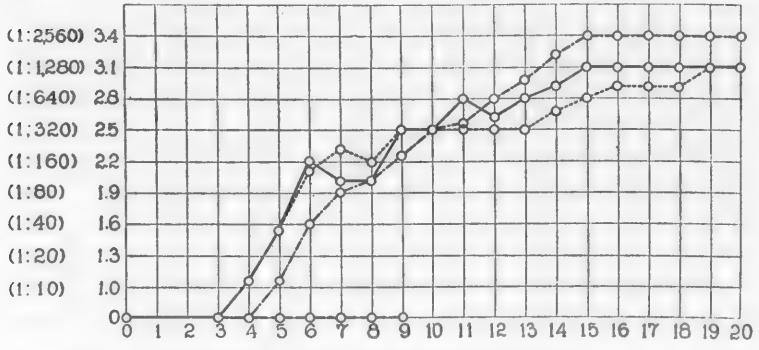
It was stated by Fabyan that the disease in guinea pigs produced by *Bacillus abortus* tended toward final recovery, although many animals might die from exhaustion and complications. These conclusions agree well with my own observations. In both his and my experiments the organism studied was isolated from cattle. Whether this is also true when the organism is isolated from other animals is a question upon which I have no data.

Inspection of all of the agglutinin curves in this and accompanying papers, except Text-fig. 2 of the present one, shows that a fall in the titer begins about the 10th week of the infection.

Two groups aggregating ten animals, the data on which are incomplete, were kept for a period exceeding 6 months after inoculation with a moderate dose of living culture of *B. abortus*. In all cases, the titer was at its height at the time of bleeding at the end of the 11th week. At the next bleeding, during the 16th week, the titer had fallen in all but one animal from the height previously occupied. During the 19th week all titers had fallen perceptibly, and at subsequent bleedings up to the time of death they were found to be falling still, slowly but definitely. When the animals were killed, after more than 6 months, only a few colonies of *B. abortus* developed in cultures from the spleen and in most cases the other organs gave no growth. It is apparent that the organisms were gradually being killed off. No animals have been kept much longer than 6 months, so that the ultimate termination of the disease is not known.

The agglutinin curves in Text-fig. 2 are exceptions to the general rule because the amount of culture used to infect was so very near the

minimum infective dose that the resultant disease was slow of onset and slow of progress. Whereas, at about the 10th week with a moderate dosage the disease would have reached its climax, in this case with a very small dose the disease was still actively progressive.



TEXT-FIG. 2. Agglutination curves of Group V guinea pigs.

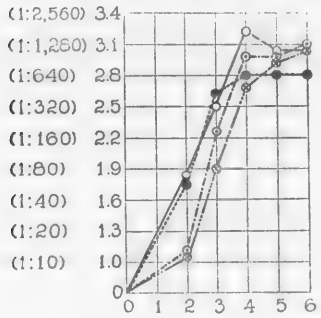
o———o Guinea Pig 2059.
o.—.—.—.—o Guinea Pig 2058.
o.....o Guinea Pig 2055.
o----------o Guinea Pigs 2056 and 2060.

The Alteration of Culture Virulence.

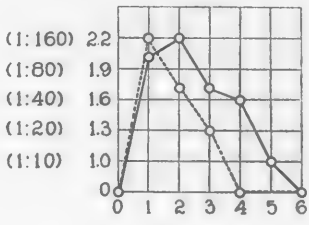
All of the work reported in this paper was done with a single strain of *Bacillus abortus* isolated less than 6 months previously. A comparison of Text-figs. 2 and 3, however, will show that enormous differences in virulence existed at different times. This difference in virulence is not simply attenuation due to *in vitro* cultivation, for Text-fig. 2, in which the virulence seems to be low, illustrates the effects of a younger cultural generation of the organism than does Text-fig. 3. This variation in virulence at different times was always encountered when dealing with small doses, so that the results in one series of animals could never be exactly duplicated in another series. It was clearly due to the influence of the bodies of the guinea pigs through which the culture had been passed.

For instance, the group represented in Text-fig. 2 was inoculated with a culture which had just been passed through Guinea Pig 1958. This animal had been

inoculated 8 weeks previous to sacrifice with a 1:1,000 dilution of a standard suspension. Only moderately severe lesions had developed, showing that the animal had been quite resistant to the infecting organism. Text-fig. 3 represents the findings in a group of animals which had been inoculated with smaller doses than those of Text-fig. 2; but the culture had just been recovered from the heart's blood of a mouse which had died within 24 hours after infection. Tracing one generation further back, it is found that the mouse was inoculated with the strain immediately after that had been recovered from the spleen of a guinea pig killed 1 week after inoculation with a large dose of culture, and that this animal had



TEXT-FIG. 3.



TEXT-FIG. 4.

TEXT-FIG. 3. Agglutination curves of Group V_3 guinea pigs.

o———o Guinea Pigs 2233 and 2232.
•·····• Guinea Pigs 2230 and 2231.
•·-·-·-• Guinea Pigs 2228 and 2229.
•—···—• Guinea Pigs 2226 and 2229.

TEXT-FIG. 4. Agglutination curves of guinea pigs receiving a single injection of killed *B. abortus* culture.

o———o Guinea Pig 2118.
o·····o Guinea Pig 2051.

exhibited marked lesions, especially great splenic enlargement. The organism thus had been passed through two animals immediately before use in the series. This treatment had apparently exalted the virulence to a very considerable degree. A third group of animals serves to demonstrate the same point. This group (Table I, Group V_2) was inoculated with the same amounts of suspension as in Group V. The agglutination curve, however, instead of resembling that of Group V (Text-fig. 2), was very steep, resembling that for Group V_3 (Text-fig. 3). In this case the culture had just been recovered from a guinea pig which had been inoculated with a large dose of *B. abortus* and killed in 1 week.

The Agglutinin Production of Bacillus abortus for Guinea Pigs.

Normal guinea pig serum will cause clumping of suspensions of *Bacillus abortus* only when in very low dilutions. Clumping in a dilution as high as 1:10 may be considered as evidence of artificial stimulation. This is easily accomplished by the inoculation of either living or dead organisms, but the former are much the more efficient.

Text-fig. 4 represents the agglutinin response of two normal guinea pigs to heavy doses of killed *B. abortus* suspension injected intraperitoneally. Comparison with Text-fig. 1 shows that the initial response is approximately the same as with heavy doses of living suspensions. After the 1st week, however, the curve falls, in the case of the dead culture, and within a few weeks has reached normal. If additional injections of dead culture are given, the response is slight and a titer of about 1:640 appears to be near the limit of their stimulation capabilities.

The administration of living cultures in quantities great enough to infect causes the agglutinin titer to rise to a height of 1:1,280 to 1:5,120, depending on the individual. Partial reactions in a dilution of 1:10,240 have been obtained in the case of a few individuals but this is quite rare.

Individual variation in the form of the agglutinin curves is very great, so that it cannot be predicted accurately. In general, however, the larger the initial dose the steeper is the first part of the curve. The ultimate height reached is the same, irrespective of the dose used.

Permeability of the guinea pig placenta to *Bacillus abortus* antibodies was demonstrated in two cases by the detection of agglutinins in the blood of fetuses taken from the uteri of infected mothers, the tissues of the fetus proving sterile upon adequate culture. In one case the titer of the mother was 1:1,280 and that of her fetus 1:160; in the other, that of the mother was 1:2,560, and that of the fetus 1:80. The fetal fluids were negative in a dilution of 1:20 in each case.

Natural Infection of Guinea Pigs with Bacillus abortus.

In the course of this work many guinea pigs, some normal and some infected, were kept together in large pens, and not a single instance of natural infection of the normal animals occurred. The animals were always of the same sex, as the two sexes were kept separate to prevent breeding during the experiment. A number of infected females left over from another experiment were divided into

two groups of five animals each and each group penned with a normal male. After 4 months, one group was killed. Four of the females were pregnant and the male was healthy. In the other group, agglutination tests at the end of the 3rd month indicated developing infection in the male. The group was sacrificed at the end of the 4th month and the following facts noted. There were no gross lesions to indicate the disease in the male. Cultures from the spleen yielded a few colonies, and those from the right epididymis showed heavy growths of *Bacillus abortus*. Cultures from other organs gave no growth. The agglutinin titer at death was 1:320. Three of the females were pregnant. Of the other two females, one was interesting as being the probable source of infection of the male animal. Aside from the usual lesions found in advanced cases of the disease (the animal had been inoculated about 6 months previously), there were two abscesses located in the wall of the uterus, one of which was discharging into its lumen. The left uterine horn was bound down by adhesions to surrounding tissues. The abscesses were old, thick walled, and filled with a thick, yellowish white, non-odorous pus, such as is frequently seen in epididymal abscesses.

It is not possible to say in what manner the male guinea pig became infected, but the finding of a *Bacillus abortus* abscess opening into the female generative organs and the isolation of the organism from the generative organs of the male, which animal showed little evidence of the usual systemic disease, forms a formidable chain of circumstantial evidence. Infection by ingestion is improbable in the light of past experience, and is rendered more so in this particular case by the fact that normal females had been associated for months with this same group of animals without contracting infection.

The Susceptibility of Guinea Pigs to Infection by Bacillus abortus through Ingestion.

The common experience that guinea pigs infected with *Bacillus abortus* may be kept with normal animals of their own sex with little or no danger of infecting the latter suggested that normal animals are probably not highly susceptible to feeding infection. This point was tested in the following experiment.

A standard suspension of *B. abortus* was made up and from this dilutions of 1:10, 1:100, and 1:1,000. With a small bulb pipette 1 cc. of each suspension was placed directly into the mouth of young normal animals. No difficulty was experienced in getting the animals to swallow the entire amount, so the exact dosage is known for each animal.

The two animals receiving the undiluted and the 1:10 dilution of the standard suspension became infected, the other two given the higher dilutions remained well. Agglutinin production in the infected animals did not become evident until the 3rd week following infection, a lag seen among inoculated animals only in those receiving a very minute dose.

It appears from this experiment that the susceptibility of guinea pigs to feeding infection with *Bacillus abortus* is actually quite low, as had been surmised. It is computed that the ratio between the minimum infecting dose of *Bacillus abortus* by inoculation and by feeding is at least as great as 1 to 100 million.

SUMMARY AND CONCLUSIONS.

1. The infectivity of *Bacillus abortus* for guinea pigs is extremely great. It is computed that somewhat less than 100 organisms are required to infect most animals.

2. Occasional animals are encountered having much more resistance to *Bacillus abortus* infection than is possessed by the average. The most resistant animal encountered was infected by a number of organisms computed to be less than 10,000.

3. The principal effect on the character of the *Bacillus abortus* disease, caused by varying the size of the infecting dose, is a change in the time relations. Very small doses produce an infection of slower course than do larger doses, but the eventual results are practically the same.

4. Judging by the agglutination curves, guinea pigs inoculated with large or medium sized doses of *Bacillus abortus* generally begin to gain mastery over the disease about the 10th week after inoculation. At this time the agglutination curve begins to decline. As was first shown by Smillie, the numbers of bacteria in the body organs begin to decline at about the 4th week but the change is rather slow until about the 10th or 12th week when a rapid decline begins, coincidently with the fall in the agglutinin titer. When the inoculating dose is small

the course of the disease may be much prolonged. The defensive forces of the body would appear to be quite inactive until a certain stage in the disease is reached.

5. The virulence of *Bacillus abortus* cultures for guinea pigs may be raised or lowered by appropriate passage through these animals.

6. Normal guinea pig blood will not agglutinate *Bacillus abortus* in a dilution of 1:10. Immune agglutinins are easily produced by inoculations with dead or living organisms. Agglutinins for *Bacillus abortus* are capable of passing the placental filter.

7. There appears to be little or no danger of normal guinea pigs becoming infected by association with diseased animals of their own sex. One instance is reported, however, of the infection of a male animal that had cohabited with infected females.

8. The susceptibility of guinea pigs to infection through ingestion of *Bacillus abortus* is relatively slight.

BIBLIOGRAPHY.

Fabyan, M., *J. Med. Research*, 1912, xxvi, 441.
Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.
Hagan, W. A., *J. Exp. Med.*, 1922, xxxvi, 711.
Smillie, E. W., *J. Exp. Med.*, 1918, xxviii, 585.
Smith, T., and Fabyan, M., *Centr. Bakt., Ite Abt., Orig.*, 1911–12, lxi, 549.

THE VALUE OF HEAT-KILLED CULTURES FOR THE PREVENTION OF THE BACILLUS ABORTUS INOCULATION DISEASE OF GUINEA PIGS.

By WILLIAM A. HAGAN, D.V.M.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, July 7, 1922.)

The great economic importance of infectious abortion in cattle and swine, particularly in cattle, has provided the stimulus for many experiments aimed at the prevention of the disease through the use of preparations made from the *Bacillus abortus* and its products. Naturally the use of killed cultures of the organism was among the first of these procedures attempted and it has, perhaps, been the method most extensively tried. In spite of this the exact immunizing value of the killed cultures is not known though it is quite generally thought at the present time to be slight. The interpretation of results of immunization experiments against infectious abortion in cattle is attended with certain inherent difficulties which have left nearly all such experiments open to question.

The disease of guinea pigs due to inoculation with *Bacillus abortus*, first described by Smith and Fabyan (1912), seemed to offer a more favorable opportunity for studying the mechanism of the process of immunity than the cattle disease, since it is not attended with the difficulties found in the latter. Such a study was taken up, therefore, not only for the light which might be cast by it on the general biology of the organism but for the possible assistance which it might render toward solving the cattle problem. It was realized, of course, that results obtained in working with one species of animal cannot safely be applied to another species, by inference, unless there is strong corroboratory evidence.

HISTORICAL.

Previous attempts to immunize guinea pigs to *B. abortus* by the use of killed cultures have been made by Ascoli (1915), and by Stafseth (1920). Stafseth found that no immunity was produced by the procedure, but his work is of little value because the size of his infecting dose was so enormous as to overwhelm any but the strongest of immunities.

Ascoli's work may be criticized similarly, although it is evident that he appreciated the importance of a reasonably small infecting dose and made an effort to attain it. His immunizing cultures were killed with ether. Both single large doses of the immunizing suspension and small repeated doses were tried. The infecting doses varied from one series of animals to another, but the smallest used was 1:100,000 of an agar slant growth. The animals were killed after periods of time varying from a few days to several months. The agglutinin titer of the blood was determined when the animals were killed, and cultures were made from the spleen. The nature and extent of the lesions apparently were not considered.

The animals previously subjected to the immunization process as well as normal animals used as controls developed agglutinins for *B. abortus* and the organism was recovered from the spleens of all. It was concluded, therefore, that the use of killed cultures of *B. abortus* was ineffectual in preventing subsequent infection by inoculation.

Plan of Experiment.

Since previous work, supported by similar observations dealing with the disease in cattle, indicated that the degree of immunity produced in the animals probably would be slight, if not nil, it was planned to carry on observations upon a quantitative basis in an effort to detect immune processes which might not be of sufficient degree to afford complete protection.

A group of twenty-four female animals was selected from the stock, uniformity of age and size being sought. All were young, half grown animals weighing in the neighborhood of 350 gm. Agglutination tests with *Bacillus abortus* antigen showed the blood of all to be negative in a dilution of 1:10. One-half of the animals (twelve) were subjected to preliminary injections of killed cultures and finally all, with the exception of two animals from each group which were preserved as controls, were inoculated with living culture. Throughout the experiment all animals were kept together in a single large pen.

Evidences of immunity in the group of animals receiving the pre-

liminary treatment were sought by comparing the group with the untreated in the following respects: (*a*) changes in body weight; (*b*) differences in the agglutinin curves; (*c*) extent and character of the lesions, with especial reference to the size and condition of the spleen; and (*d*) number of *Bacillus abortus* cultivable from the spleen.

Immunization.

The culture used throughout this work was a typical strain of *B. abortus* isolated from a bovine placenta, Case A 898, by passage through Guinea Pig 1865. It grew readily on plain veal infusion agar, provided the cultures were hermetically sealed. For antigenic material this strain was grown 48 hours on plain agar in a Blake flask which had been sealed with wax. A suspension of the resulting growth was made in physiological salt solution and heated at 60°C. for 1 hour. Both cultural and animal inoculation tests proved the suspension to have been sterilized. It was diluted to a reading of 2.4 cm. on the gauge devised by Gates (1920) and stored, without preservation, in the refrigerator until ready for use.

The twelve animals selected for immunization were given intraperitoneal injections of 1 cc. of the killed bacterial suspension weekly. The manner in which the animals were reacting to the injections was gauged by the agglutination curve. The weekly injection of rather large doses of killed bacterial suspension appeared to exert little effect on the health of the animals, although reference to Table I will show that the group did not gain in weight so well as did the untreated group. The gain in weight during the period of immunization for the treated group averaged 162 gm., while for the other group the average was 231 gm.

The curve in Text-fig. 1 is typical of the agglutinin response in all of the treated animals. After a sudden rise following the first injection there was a slow rise until an average titer of 1:640 was reached, beyond which point it appears that there is no stimulation by the use of dead cultures. After six weekly injections the process was stopped and some time was allowed to elapse before the test inoculations were made, in order to allow the body to recover its equilibrium which it was thought might have been upset by the stress of the immunizing process. At the end of the 3rd week following the last dose of killed culture, the agglutinin titers began to show evidence of falling and the time was considered ripe for inoculation with the living culture.

The Infecting Dose.

The strain of *Bacillus abortus* which had been used in the preparation of the immunizing suspension was also used for infection.

The culture had been passed through Guinea Pig 2106 just prior to use. The growth from a 24 hour sealed slant agar culture was washed off with physiological salt solution and the resulting suspension diluted to make a reading of 2.4 cm. on the Gates loop gauge. This makes a suspension somewhat denser than a 24 hour typhoid culture in bouillon. Dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} were made from the original suspension. The last three dilutions were used for inoculating, 1 cc. being injected intraperitoneally in each case.

In the matter of dosage, it was sought, in some of the animals, to approach rather closely to the minimum amount necessary to produce infection; in others to give a moderate dose; and in a few to give what might be considered a comparatively large dose. The question of the minimum infective dosage of suspensions of *B. abortus* is discussed elsewhere (Hagan, 1922). Reference to the communication cited will show that the smallest dose administered was probably several times the amount actually needed to produce infection. The individual variation in resistance is so great that smaller doses than the minimum amount given in this experiment could not safely be depended on to infect all normal animals, although $\frac{1}{100}$ of this amount probably would have infected most guinea pigs.

The animals were killed and examined in pairs consisting of one animal from each group. Some were killed within 3 weeks after the test inoculation; others in 6, 9, and 12 weeks. All were bled regularly at weekly intervals and the agglutination curves obtained.

RESULTS.

Body Weight.—A complete weight record will be found in Table I. Analysis of this shows that during the entire experiment the average gain in body weight was approximately the same in the two groups; *i.e.*, immunized group 308 gm., non-immunized 304 gm. At the same time two animals used as controls, which were not subjected to any experimentation except periodic bleeding for agglutination work, made an average gain of 430 gm. It has already been stated that during the immunization process the treated animals were outstripped in the amount of weight gained by some 70 gm. on the average for the group. From the time of inoculation to the time of death the position was reversed, the immunized animals gaining 109.5 gm. while the non-immunized gained only 53 gm. This margin undoubtedly would have been widened if the animals had been allowed to live longer, as

is indicated by the comparatively great weight losses suffered by the non-immunized animals which were allowed to live the longest.

The Agglutination Curves.—At the time of administering the living culture, the agglutinin titer of the immunized group averaged about 1:320, while the bloods of the non-immunized were negative at a dilution of 1:10. Text-fig. 1 shows the behavior in the immunized control animals. Following the last injection of killed culture there is a gradual decline in the curve lasting some 13 weeks before it reaches the base-line. Text-figs. 2 to 4 show the curves in the inoculated animals receiving different amounts of infective material. The latter halves of these curves show that there is practically no difference in the agglutinin response to the infecting dose in the two groups, and that the size of the infecting dose has made little difference in the agglutinin response. In Text-figs. 3 and 4 the smaller doses have produced a delay in the agglutinin production, and the curve for the immunized group falls appreciably before the effect of the test inoculation becomes operative. In the case of the comparatively heavy inoculation represented by Text-fig. 2 this fall does not occur.

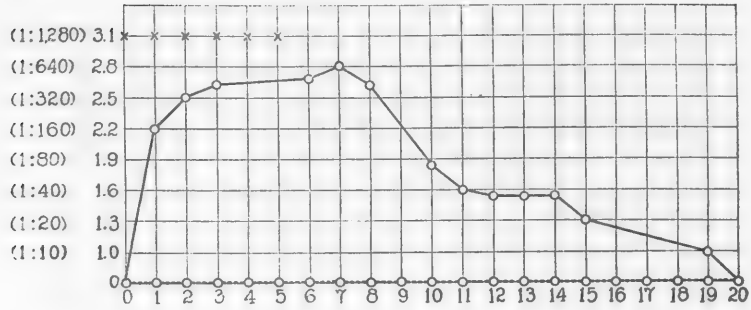
Differences in Extent and Character of Lesions.—All of the inoculated animals became infected, thus showing that the treatment was ineffectual in preventing the disease. Certain differences in the character of the disease process have been noted, however, as a result of the preliminary treatment. These have to do with the spleen, lymph glands, and kidneys.

The spleen presented the most striking lesion of the disease in all cases, immunized or non-immunized. Reference to the spleen weights in Table I shows that enlargement occurred in all cases except in Guinea Pigs 2083 and 2071, both of which were immunized animals which had received small doses of the infecting culture and were killed early. The splenic enlargement, with one exception, was greater, however, and usually much greater in the non-immunized than in the immunized group. The spleen weight for the former group averaged 4.87 gm., while for the latter it was only 3.1 gm., a difference of more than 50 per cent. There is no evidence that the character of the process in the spleen is different in the two groups. It is probable that the difference is only a matter of degree; *i.e.*, that in the immunized group the process is only delayed.

TABLE I.

Guinea pig No.	Immunized.	Injected.	Length of time lived.	Initial weight.	Maximum weight.	Weight when killed.	Loss in weight from maximum.	Change in weight during immunization.	Change in weight during disease.	Spleen weight.	Agglutinin titer when killed.	Spleen.	
												Bacteria per gm.	Bacteria per organ.
			days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*			
2073	+	Control.	24	410	720	720	0	+190	+100	1.07	1:40	0	0
2081	−	"	24	405	740	740	0	+215	+ 95	1.06	0	0	0
2074	+	10^{-3}	24	390	670	670	0	+155	+ 90	4.12	1:5,120	?	?
2082	−	10^{-3}	24	410	745	725	20	+245	+ 70	7.34	1:1,280	807,000	5,900,000
2083	+	10^{-5}	24	370	690	690	0	+170	+110	1.07	1:160	83,000	88,000
2090	−	10^{-5}	24	430	680	680	0	+190	+ 70	3.09	1:640	43,000	124,000
2071	+	10^{-6}	24	335	710	710	0	+185	+130	1.19	1:320	615,000	731,000
2076	−	10^{-6}	24	465	755	720	35	+250	− 10	6.01	1:320	367,000	2,205,000
2070	+	10^{-5}	42	340	645	645	0	+ 75	+110	3.00	1:2,560	123,000	369,000
2077	−	10^{-5}	42	395	640	530	110	+200	− 85	5.49	1:5,120	196,000	1,078,000
2067	+	10^{-6}	42	370	660	630	30	+160	+100	2.48	1:1,280	198,000	491,000
2080	−	10^{-6}	42	385	760	760	0	+235	+130	2.59	1:1,280	42,000	109,000
2086	+	10^{-5}	63	400	750	750	0	+220	+ 90	2.24	1:1,280	12,500	38,000
2088	−	10^{-5}	63	430	1,000	1,000	0	+240	+300	2.23	1:2,560	?	?
2075	+	10^{-6}	63	410	850	850	0	+180	+240	4.8	1:2,560	33,800	162,000
2079	−	10^{-6}	63	305	725	725	0	+215	+180	2.74	1:5,120	?	?

2068	+	Control.	85	415	800	800	0	+160	+170	1.07	0	0	0
2085	−	"	85	385	915	915	0	+260	+235	1.07	0	0	0
2069	+	10^{-3}	85	335	560	480	80	+125	− 10	5.92	1:5,120	109,000	645,000
2078	−	10^{-3}	85	390	710	540	170	+230	−115	8.75	1:5,120	75,500	662,000
2072	+	10^{-5}	85	365	730	730	0	+185	+155	3.26	1:2,560	11,300	36,900
2087	−	10^{-5}	85	390	915	670	245	+260	− 30	4.32	1:2,560	38,000	105,000
2084	+	10^{-6}	85	330	600	570	30	+140	+ 80	2.97	1:2,560	43,000	128,000
2089	−	10^{-6}	85	400	830	690	140	+230	+ 20	6.17	1:5,120	58,600	361,500

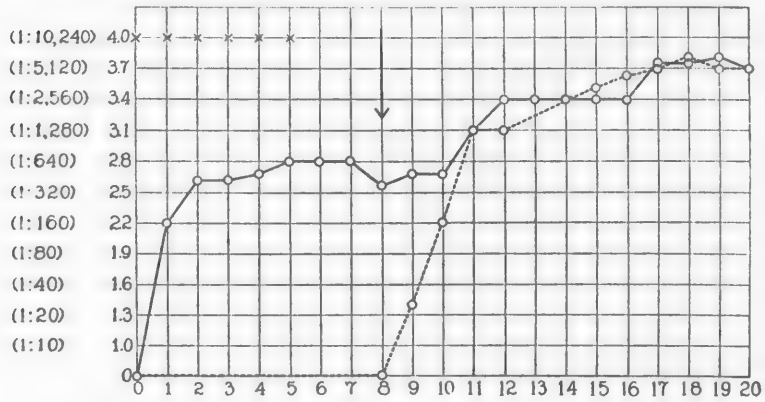


TEXT-FIG. 1. Composite agglutinin curves of normal control, and of immunized but non-infected animals.

o———o Composite curve of Guinea Pigs 2068 and 2073, animals which were immunized but not infected. The times of injecting the killed suspensions of *B. abortus* are indicated at the points x.

o·····o Composite curve of Guinea Pigs 2081 and 2085, animals which were untreated but allowed to live throughout the experiment in the same pen with the infected animals.

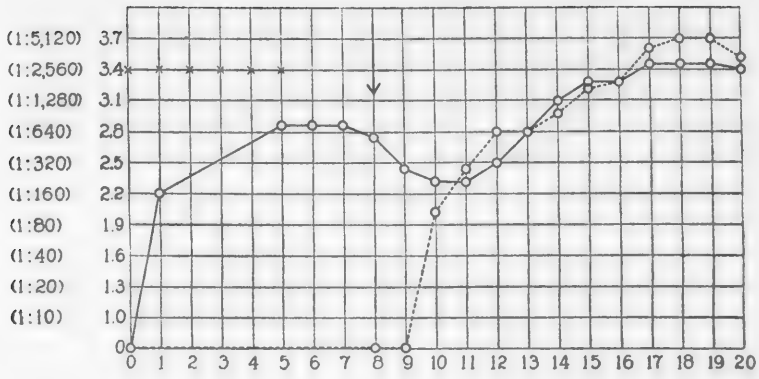
In this and all succeeding agglutinin curves, the abscissæ represent weeks, and the ordinates the logarithms of the dilution of the blood. The actual blood dilutions are given in parenthesis.



TEXT-FIG. 2. Composite agglutinin curves of immunized and non-immunized animals, infected with 1:1,000 dilution of a standard suspension of *B. abortus*. The times of injecting killed suspension of *B. abortus* are indicated by x, the time of infection by the arrow.

o———o Guinea Pigs 2068 and 2074, immunized.

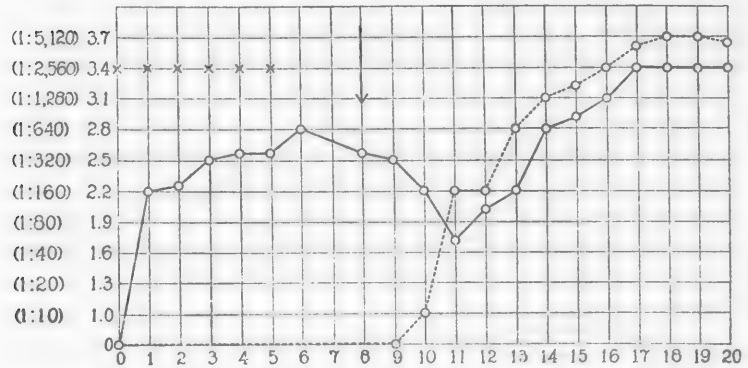
o·····o Guinea Pigs 2078 and 2082, non-immunized.



TEXT-FIG. 3. Composite agglutinin curves of immunized and non-immunized animals, infected with a 1:100,000 dilution of a standard suspension of *B. abortus*.

o———o Guinea Pigs 2070, 2072, 2083, and 2086, immunized.

o·····o Guinea Pigs 2077, 2087, 2088, and 2090, non-immunized.



TEXT-FIG. 4. Composite agglutinin curves of immunized and non-immunized animals, infected with a 1:1,000,000 dilution of a standard suspension of *B. abortus*.

o———o Guinea Pigs 2067, 2071, 2075, and 2084, immunized.

o·····o Guinea Pigs 2076, 2079, 2080, and 2089, non-immunized.

Enlargement of the lymph glands occurred, coincident with splenic enlargement, and was more pronounced in the non-immunized animals than in the immunized.

A striking lesion in the non-immunized animals which lived the longest and in several of the non-immunized animals which were killed early occurred in the kidneys. This lesion is frequently found in the later stages of the guinea pig disease and was described by Smith and Fabyan (1912). It consists in cellular infiltration in the early stages, followed later by sclerosis. That the lesion is of great import to the body economy is rendered probable by the fact that as the severity of the sclerosis increases emaciation of the body keeps pace. The condition is always accompanied by a very large spleen, but great enlargement of the spleen is sometimes found without accompanying kidney disease and emaciation. This kidney lesion was found among the immunized animals but once, in Guinea Pig 2069, one of the animals which received a heavy infecting dose.

The Number of Bacillus abortus Present in the Spleen.—The manner of determining the bacterial count of the spleen is described in the appendix. The counts are given in Table I. It will be noted that the bacterial count per gram of splenic tissue is greater in the immunized than in the other group, while the reverse is true when the bacterial content of the entire organ is considered. The explanation for this lies in the greater enlargement of the organ in the latter group. Since this enlargement is largely due to engorgement of the sinuses with blood, the bacterial concentration per unit of volume is decreased by dilution. Taking the entire organ, however, there is an appreciably greater number of cultivable *Bacillus abortus* present in the spleens of the non-immunized than in those of the immunized animals.

SUMMARY AND CONCLUSIONS.

From the data presented it seems permissible to draw the following conclusions.

1. Guinea pigs cannot be rendered immune to the *Bacillus abortus* inoculation disease by treatment with heat-killed cultures of this organism. This agrees with the work of Ascoli, and with the general conclusions of many that dead cultures confer very little or no immunity to infectious abortion in cattle.

2. The progress of the disease can be delayed appreciably by such treatment. This is supported by the following observations: (*a*) the loss in body weight due to the disease is delayed; (*b*) the development of splenic enlargement is partially inhibited or delayed; (*c*) the development of renal lesions is delayed or prevented; and (*d*) the rapidity of multiplication of *Bacillus abortus* in the splenic pulp is reduced.

APPENDIX.

Technique of Bleeding Guinea Pigs for Agglutination Tests.—The repeated bleeding of guinea pigs for periodic agglutination tests has been accomplished in an easy and fully satisfactory manner. Many animals have been bled ten to twenty times without injury and without difficulty in obtaining the requisite amount of blood for macroscopic tube tests. The method is as follows:

The laboratory should be warm so as to encourage the circulation in the peripheral vessels. One of the ears is rubbed briskly with a pledget of cotton soaked in xylol, and then dried with a clean bit of cotton; the blood vessels are examined and one of them nicked transversely with the point of a scalpel. The vessels can be seen to better advantage if some form of illumination below the ear is used. A vigorous flow of blood generally wells up from the small incision. The blood is taken into a 0.1 cc. serological pipette which is provided, for convenience, with a length of rubber tubing and a mouthpiece. One pipetteful (0.1 cc.) is immediately delivered into 0.9 cc. of citrated physiological salt solution, making a dilution of the blood of approximately 1:10. If necessary to work with low dilutions, two pipettefuls (0.2 cc.) may be delivered into 0.8 cc. of salt solution, making a dilution of 1:5. The tubes are shaken up and placed in the refrigerator over night. The next day as much as 0.7 to 0.8 cc. of a perfectly clear dilution of plasma may be pipetted off.[1] Since only 0.5 cc. is needed for the agglutination tests, the quantity is ample. The citrate-salt solution consists of 0.9 per cent sodium chloride and 2.0 per cent sodium citrate in distilled water. This amount of citrate

[1] The dilution figures in this and accompanying papers are given in terms of the whole blood. The serum or plasma dilutions may be roughly computed as twice as great as that of the whole blood.

does not have any effect on the agglutinin titer so far as could be determined by checking against sera obtained by defibrination. The citrated plasma has the advantage over most sera in that it is perfectly clear and untinged with hemoglobin.

Agglutination Technique.—The technique of carrying out the agglutination tests is practically that described by Smillie, Little, and Florence (1919).

The antigen has been freshly prepared and no preservative added, although equally good results appeared to be obtained by using an antigen preserved with 0.25 per cent formalin and kept stored in the refrigerator for several months. Antigen preserved with 0.5 per cent phenol is not satisfactory because it induces a permanent cloudiness of the suspension which renders the reading of partial reactions difficult.

The Determination of the Approximate Numbers of Bacillus abortus Present in the Spleen.

Preparation of Splenic Tissue for Plating.—The following method was used for obtaining a measured and representative sample of splenic tissue for plating purposes.

A portion of the organ representing from 0.5 to 1.5 gm. is removed aseptically to a sterile weighed potato tube and its weight determined. The fragment is then crushed thoroughly in a manner to be described and diluted with nine times its weight of physiological salt solution. After thorough mixing, 0.1 cc. portions are used for plating, unless it is suspected that the bacterial count will be greater than 100,000 organisms per gm. of tissue, in which case 0.1 cc. of a 1:5 or 1:10 dilution of the original suspension is used.

For grinding or crushing the splenic tissue efficient crushers were made from heavy walled culture tubes in the following manner.

Tubes of as nearly perfect form as possible are selected from the stock and arranged in pairs, one tube of which is of such size that it will just slip easily into the other like the plunger of a syringe. The smaller tube is then constricted in its middle part by heating in a blast flame and drawing it out. The larger tube is shortened by cutting it off at such length that its mouth will be about at the level of the middle of the constriction of the smaller tube when the latter is placed within it. The inner tube is used as a pestle while the outer serves as

a mortar. The surface of the pestle is roughened by rotating it in contact with a piece of carborundum paper held in the hand. The inside of the mortar has not been altered although it probably could be improved somewhat by etching with hydrofluoric acid. Great roughening is not to be desired since the mortar is apt to be cut and broken, in the process of grinding, by sharp edges on the pestle. For the same reason abrasives such as fine sand or carborundum powder cannot be used. The apparatus is intended for crushing purposes rather than for grinding.

The crusher is wrapped in paper and sterilized by hot air. The piece of weighed spleen is placed in the larger tube and forced to the bottom with the pestle. It is now compressed and broken up by rotating and exerting gentle pressure on the pestle, the soft pulp being forced up between the sides of the pestle and the inner wall of the mortar. If the fit between the two elements of the crusher is good, by the time the pulp reaches the space in the upper end of the mortar left by the constriction of the shaft of the pestle it will be thoroughly reduced to a homogeneous paste. When the crushing has been completed small portions of salt solution are pipetted into the mortar and the pestle slowly lifted while being rotated. The vacuum in the bottom of the pestle causes the fluid to pass the pestle, thereby cleansing it of the pasty pulp mass. Several repetitions of this process generally leave the pestle clean, after which it is removed and the remainder of the quantity of salt solution needed to make a 1:10 dilution added.

Plating Methods.—With *B. abortus* ordinary plating methods are useless because this organism requires a peculiar gaseous environment which is not satisfied by the free atmospheric exchange of the ordinary Petri dish. For obtaining suitable gaseous conditions for the cultivation of the *B. abortus* in plate culture, there have been available the methods of Nowak (1908) and of Huddleson (1920). Since both of these are somewhat involved, while furthermore simple hermetic sealing of the tubes had proven an entirely satisfactory method of obtaining growth in tube cultures, it was decided to try this method with plate cultures in lieu of the others. A simple and satisfactory method of sealing the plates has been devised and the results in obtaining growth of *B. abortus* have been entirely satisfactory.

Pieces of double strength window glass were cut into 12 cm. squares, and others into pieces 12 by 24 cm. in order to handle two cultures as a unit. The edges of the glass were ground off to remove sharp points and corners. These squares were used as bases upon which the halves of the Petri dishes containing the cultures were sealed. The steps in the method are as follows:

1. Paraffin of a melting point of 55–60°C. is placed in a shallow vessel and heated until it smokes. The high temperature serves to sterilize the paraffin. When it has cooled to 60–70°C., a few degrees above its melting point, it is ready for use.

2. A glass square is placed on the table and its upper surface thoroughly traversed with the Bunsen flame. This not only sterilizes the surface but serves to warm the glass.

3. The lower half of the Petri dish containing the inoculated agar is grasped. open surface downward, with the tips of the fingers and the edges dipped into the molten paraffin to a depth of 5 to 8 mm. The entire margin should not be submerged at one time, otherwise when the plate is lifted the rush of air under the edge is apt to cause spattering of the paraffin on the agar surface. This may be avoided easily by tilting the plate when submerging. Since the temperature of the glass of the Petri dish is lower than the melting point of the paraffin, a layer of the latter in a congealed state adheres to the plate when it is lifted.

4. The plate is placed on the center of the glass square and gently pressed down. Since the square is warm, and there has been no delay between the dipping of the edges of the plate and planting it on the glass surface, an excellent seal between the plate edge and the glass base is formed.

5. To assure a perfect seal, the flame of a micro burner is run quickly around the joint between plate and base. If done properly the surface only of the paraffin layer will be melted and all cracks and crevices will be filled. If too much heat is used the glass will become heated through and will cause the paraffin to spread out in a thin layer on the base, thus forming an imperfect seal, or at best a thin seal which is apt to rupture through when a partial vacuum has formed within the plate.

Even when hermetically sealed, poured plates are not satisfactory for quantitative work when dealing with *B. abortus* for the reason that the organism will not develop in anaerobic or semianaerobic conditions. The deep lying bacilli will, in most instances, fail to develop and those that do grow will produce colonies so minute that counting is difficult or impossible. Surface inoculation has been used exclusively in this work. 0.1 cc. of the splenic suspension is pipetted onto the surface of the agar plate and spread uniformly by means of a bent glass rod. This amount of fluid will spread evenly over the surface of the agar without leaving an excess of fluid. The plate is then tilted, in an inverted position, on the rim of the lid to allow the surface to dry somewhat before it is sealed.

Growth generally appears within 3 to 4 days, though sometimes not for 5 or 6 days. Within 24 hours of the time when colonies can first be seen, they will have reached a diameter of 1 mm. if there is not too much crowding. After this, growth is comparatively slow, but if very few colonies are present they may reach a diameter of from 3 to as much as 6 mm. The colonies have no tendency to spread or coalesce. Because of the discrete character of the growth as many as 1,000 colonies may grow on a single plate without evidence of interference with each other, other than that their ultimate size will not exceed 1 mm.

Generally all colonies appear at the same time and grow at about the same rate of speed, so that they have about the same size. In some instances, however, two crops of colonies may appear. In these cases the second crop will become visible 1 to 3 days after the first as minute colonies lying between the first. The second crop colonies always remain small, seldom reaching a diameter of 0.5 mm. even when they are not crowded and are subjected to prolonged incubation.

Because of this phenomenon, it is always advisable to incubate the plates for 3 or 4 days following the appearance of the first crop of colonies before making the colony count.

BIBLIOGRAPHY.

Ascoli, A., *Z. Infektionskrankh. Haustiere*, 1915, xvii, 156.
Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.
Hagan, W. A., *J. Exp. Med.*, 1922, xxxvi, 697.
Huddleson, I. F., *Michigan Agric. Exp. Station, Bull. 49*, 1920.
Nowak, J., *Ann. Inst. Pasteur*, 1908, xxii, 541.
Smillie, E. W., Little, R. B., and Florence, L., *J. Exp. Med.*, 1919, xxx, 341.
Smith, T., and Fabyan, M., *Centr. Bakt., Ite Abt., Orig.*, 1911–12, lxi, 549.
Stafseth, H. J., *Michigan Agric. Exp. Station, Bull. 49*, 1920.

THE SUSCEPTIBILITY OF MICE AND RATS TO INFECTION WITH BACILLUS ABORTUS.

By WILLIAM A. HAGAN, D.V.M.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, July 7, 1922.)

It was observed by Holth (1911) that an acute disease resulted when mice and rats were inoculated intraperitoneally with cultures of *B. abortus*. It was stated that from 0.25 to 0.5 cc. of a serum bouillon culture in mice, and from 0.5 to 1 cc. in rats, was sufficient to produce a prostrating disease with fatal termination in from 2 to 5 days. The disease was essentially an intoxication. At autopsy the principal lesion found was a moist, reddened peritoneum with large numbers of the specific organism and a few leucocytes on its surface. Culturally, a few organisms could be found in the blood after death.

These observations were confirmed, in general, by Zwick and Zeller (1912), and by Ascoli (1915). In Ascoli's tables, it is noted that *B. abortus* was recovered culturally in several cases, presumably from the spleen, after periods of time as great as 2 months after inoculation, when because of insufficient dosage or artificial immunization death did not occur from the acute form of the disease. Ascoli does not mention the presence or absence of pathological changes in these cases. Zwick and Zeller noted an occasional acute swelling of the spleen, in addition to the changes described by Holth.

Fabyan (1912) showed that a type of disease entirely different from that just described might be produced in mice by intraperitoneal or subcutaneous injection of *B. abortus*. This disease was of a chronic nature, similar in many respects to that produced by the organism in guinea pigs. Although his dosage was rather high he does not mention having observed the acute disease previously described. It is possible that his statement that "many of the animals died from complications, frequently without apparent cause" refers to this form of the disease. 3 to 4 weeks after inoculation he found the spleens enlarged in thirteen of nineteen animals which had survived from a group of about thirty inoculated. Lesions in the spleen, lungs, liver, kidneys, lymph glands, and epididymis were found. Rats were inoculated also but no effect of the inoculations was detected.

INTRODUCTION.

The work just described made it clear that mice and rats could be infected experimentally with *Bacillus abortus* when a large amount of

infective material was employed for inoculation. Whether these animals could be infected with such small amounts as to make them useful in detecting the organism in diagnostic work with infectious abortion of cattle, and whether they were sufficiently susceptible to infection by ingestion to make the wild varieties a possible factor in the propagation and transmission of the disease among cattle were questions unsolved. To the working out of these two questions this paper is devoted.

The Susceptibility of White Mice to the Inoculation Disease.

Ten young, white, male mice of about the same size and age were selected from the stock and divided into five pairs, each pair being placed in a jar by themselves. The animals of the first pair were inoculated differently, one with a 48 hour serum bouillon culture of *Bacillus abortus*, and the other with an undiluted suspension of *Bacillus abortus* washed from a 48 hour agar slant culture. The second pair received a 10^{-3}, the third a 10^{-4}, the fourth a 10^{-5}, and the fifth a 10^{-6} dilution of this suspension. All were inoculated with 0.5 cc. of dilution, intraperitoneally. One guinea pig was inoculated with 0.5 cc. of the 10^{-6} dilution as a check on the virulence of the culture and for purposes of comparison.

The suspension above referred to was made by growing a bovine strain of *Bacillus abortus*, recently recovered from a lymph gland of Guinea Pig 2082, in a sealed tube of plain veal infusion agar for 48 hours, then making a suspension of the growth in sterile physiological salt solution, and diluting this to a reading of 2.4 cm. on the standardizing gauge of Gates (1920).

The results of the experiment are recorded in Table I. A number of the animals died early from an intercurrent pneumonia. The results sought, however, were obtained in a clear-cut manner. All of the animals became infected as evidenced by the recovery of the organism from the spleens. The cultural record for Animal C was misplaced and lost, and decomposition prevented cultural work on Animal B.

Table I shows clearly that white mice may be infected with very minute amounts of cultures of *Bacillus abortus*. The results with the guinea pig indicate that mouse susceptibility to infection with

TABLE I.

White Mice.

Mouse.	Infecting dose.	Autopsied.	Spleen culture.	Remarks.
A	0.5 cc. serum bouillon culture.	Died; 27th day.	Positive, moderate.	Died from pneumonia. *Klossiella* infection of kidneys.
B	Suspension 0.5 cc. undiluted.	" 3rd "	None made.	Badly decomposed. Died from pneumonia. No evidence of peritonitis.
C	" 0.5 " 1:1,000 dilution.	" 9th "	Record lost.	Purulent pneumonia.
D	" 0.5 " 1:1,000 "	Killed; 27th "	Positive, luxuriant.	Spleen enlarged × 3. Other organs normal. Agglutinins 1:160.
E	" 0.5 " 1:10,000 "	Died; 26th "	" moderate.	Spleen normal. Slight pneumonia. *Klossiella* infection of kidneys.
F	" 0.5 " 1:10,000 "	Killed; 27th "	" luxuriant.	Spleen enlarged × 3. Slight pneumonia. Agglutinins 1:320.
G	" 0.5 " 1:100,000 "	Died; 11th "	" good.	Kiiied by fighting. Slight splenic enlargement. Slight pneumonia. *Klossiella* infection.
H	" 0.5 " 1:100,000 "	Killed; 27th "	" luxuriant.	Spleen enlarged × 3. Slight pneumonia. Other organs normal.
I	" 0.5 " 1:1,000,000 "	" 27th "	" "	Spleen normal size. Slight pneumonia. Agglutinins 1:40.
J	" 0.5 " 1:1,000,000 "	Died; 2nd "	" very few colonies.	Wounded by fighting. Very weak when inoculated. No lesions of internal organs.
Guinea Pig 2114	" 0.5 " 1:1,000,000 "	Killed; 27th "	Positive, moderate.	No lesions. Agglutinins 1:40.

this organism compares favorably with that of the latter animal. No experiments have been done with infected tissues, but there is no apparent reason why white mice should not serve successfully as inexpensive substitutes for guinea pigs in diagnostic work on infectious abortion of cattle.

The Susceptibility of Rats and Mice to Bacillus abortus by Ingestion.

The susceptibility of both wild and tame varieties of mice and rats to infection by feeding with *Bacillus abortus* was tested. Cultures and infected tissues were used.

TABLE II.

White Rats.

Rat.	Method of infection.	Material used.	Autopsied.	Spleen culture.	Agglutinins.	Remarks.
			day			
A	Feeding.	Culture.	52nd	−	−	
B	"	Tissue.	52nd	−	−	
C	Inoculation.	Culture.	23rd	+	1:40	No lesions.
D	"	" 1:100 dilution.	26th	+	1:40	" "
E	"	"	52nd	+	1:40	" "
F	Control.	−	52nd	−	−	

The cultures were salt solution suspensions of the growth removed from agar slants which had been incubated for 24 to 48 hours. Approximately 3 cc. of salt solution was used to suspend the growth from a single tube culture. The infected material was the livers of guinea pigs which had been infected with *B. abortus* and killed during the 4th week after inoculation. Although the organs of the body of the guinea pig are richest in the abortion bacilli at this time, the liver is never the seat of large numbers of the organism. The livers used for feeding had all yielded the *B. abortus* in moderate numbers.

The animals were allowed to fast for 24 hours before feeding. The culture suspensions were placed in Petri dishes and small bits of dry bread added to absorb the fluid. When offered to the hungry animals, the soaked bread was always eagerly consumed. The livers were ground to a pasty consistency in a mortar and smeared on pieces of bread. The paste was eagerly licked off the bread by the rats and taken fairly well by the mice. The day after the first feeding food was again withheld, and on the 3rd day the feeding of infected material was repeated as before and with the same materials.

The results of the feeding experiments are given in Tables II to VI inclusive. In each case several animals were inoculated subcutaneously as controls. It will be noted that all inoculated animals became infected, while all of the feeding experiments, with two exceptions, failed. The two exceptions, one a gray rat (*cf.* Table IV, Rat B), and one a white rat (*cf.* Table III, Rat B), received heavy culture feedings. The data on gray rats are limited because of the difficulty of procuring the animals. In Table III, however, it is

TABLE III.

White Rats.

Rat.	Material fed.*	Autopsied.	Spleen culture.	Agglutinins.	Remarks.
		day			
A	Culture suspension, undiluted, 1 cc.	22nd	Cultures contaminated.	1:10	Died from pneumonia. Organs invaded with colon bacilli.
B	Culture suspension, undiluted, 1 cc.	29th	+	−	Very few organisms present in spleen. No lesions.
C	Suspension, 1:10 dilution, 1 cc.	29th	−	−	
D	Suspension, 1:100 dilution, 1 cc.	29th	−	−	
E	Suspension, 1:1,000 dilution, 1 cc.	29th	−	−	

* In this case the bacterial suspensions were pipetted directly into the mouths of the animals, so the exact dosage is known.

TABLE IV.

Gray Rats.

Rat.	Method of infection.	Material used.	Autopsied.	Spleen culture.	Remarks.
			day		
A	Feeding.	Infected tissue.	19th	−	
B	"	Culture.	19th	+	No lesions. Organism recovered from spleen.
C	Inoculation.	" suspension 1 cc.	22nd	+	No lesions. Organism recovered from spleen. Agglutinins 1:1,280.

demonstrated that the white rat becomes infected by feeding, only if he receives a very heavy dose of the organism; and this is probably also true of the gray rat. The failure, in all cases, of the guinea pig tissue to infect is best explained on this basis.

TABLE V.

White Mice.

Mouse.	Method of infection.	Material used.	Autopsied.	Spleen culture.	Agglutinins.	Remarks.
			day			
A	Feeding.	Infected tissue.	52nd	—	—	
B	"	Culture.	52nd	—	—	
C	"	"	28th	—	—	
D	"	"	28th	—	—	
E	Inoculation.	"	23rd	+	1:20	Enlarged spleen.
F	"	"	52nd	+	—	" "
G	"	" 1:100 dilution.	26th	+	—	" "
H	Control.	—	52nd	—	—	

TABLE VI.

Gray Mice.

Mouse.	Method of infection.	Material used.	Autopsied.	Spleen culture.	Agglutinins.	Remarks.
			day			
A	Feeding.	Culture.	28th	—	—	
B	"	"	28th	—	—	
C	"	"	2nd	—	—	
D	"	"	33rd	—	—	
E	"	"	33rd	—	—	
F	"	"	52nd	—	—	
G	"	Infected tissue.	52nd	—	—	
H	Inoculation.	Culture.	21st	+	1:160	Organs normal.
I	"	"	52nd	+	1:20	" "
J	"	" 1:100 dilution.	52nd	+	—	" "

CONCLUSIONS.

1. White mice are highly susceptible to infection by inoculation with *Bacillus abortus*. The susceptibility appears to be as great as that of the guinea pig, and this animal probably can be substituted satisfactorily for guinea pigs in diagnostic work.

2. Both mice and rats are very refractory to feeding infection with *Bacillus abortus*. The failure to infect mice in this way was complete, but the feeding of large amounts of culture gave infection in rats. Subcutaneous inoculation resulted in infection of all the animals. The difficulty of infecting rats and mice with *Bacillus abortus* by feeding makes it very doubtful whether these animals can have any rôle in the propagation and spread of infectious abortion in cattle.

BIBLIOGRAPHY.

Ascoli, A., *Z. Infektionskrankh. Haustiere*, 1915, xvii, 156.
Fabyan, M., *J. Med. Research*, 1912, xxvi, 441.
Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.
Holth, H., *Z. Infektionskrankh. Haustiere*, 1911, x, 207.
Zwick and Zeller, *Arb. k. Gsndhtsamte*, 1912–13, xliii, 1.

THE ACTION OF SODIUM SALICYLATE UPON THE FORMATION OF IMMUNE BODIES.

By HOMER F. SWIFT, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 7, 1922.)

INTRODUCTION.

This study is one of a series to determine the action of the salicylates upon some of the manifestations of infection in the animal body. From a pharmacological view-point it is well established that the salicylates have an antipyretic and analgesic action in patients when administered in therapeutic doses. The analgesic action varies in different individuals and is apparently more powerful with some of the salicylate compounds than with others. This effect is obtained in many conditions, both in the general toxic state seen in most infections, and also in neuralgias and myalgias when indefinite pain is the chief symptom, and fever and other general manifestations of infectious intoxication are usually absent.

The antipyretic action is due to an increased loss of body heat and, as shown by Barbour (1), is evidenced almost exclusively by individuals who are suffering from infection; normal persons show little if any antipyretic effect after taking fairly large doses of the drug. Afebrile patients suffering from infections, on the other hand, show an increased heat loss after receiving comparatively small doses of the drug. It seems as though the heat-regulating center of this class of individuals is in a different state of sensitiveness to salicylates than is that of normal persons.

A third action of salicylates demonstrated almost exclusively in patients with the polyarthritis of rheumatic fever is that of an antiphlogistic agent. Following the administration of the drug in amounts just under the toxic dose, a most striking decrease of local heat, redness, and swelling, as well as of pain and tenderness in the

involved joints is usually observed; and, in addition, the tendency for the inflammation to spread to other joints is arrested. Patients suffering from gonorrheal arthritis or other joint infections in which bacteria can be demonstrated in the arthritic fluid are not benefited in this striking manner by salicylates. So often has this observation been repeated that the therapeutic test is frequently applied to differentiate the acute polyarthritis of rheumatic fever from that due to other causes. It seems fairly well established, on the other hand, that the duration of the general infection in rheumatic fever is not materially shortened by the drug, and that cardiac complications often arise even though the patients are taking amounts sufficient to keep their temperatures almost normal and to prevent arthritis recurring. From clinical observation alone it would seem, therefore, that the drug either lowered the infectivity of the etiologic agent of rheumatic fever by a direct action upon the virus or increased the ability of the body to react against the infection.

It is well known that salicylic acid and sodium salicylate have an antiseptic action *in vitro*; this action is much stronger with free salicylic acid than with its salts. It is, moreover, impossible for the free acid to occur in the tissues or body fluids in sufficient concentration to exert any marked bactericidal action. A bacteriostatic action must be considered; the drug may exist in sufficient concentration or in a state to inhibit the growth of the infectious agent, even though the virus is not completely killed. A study of this problem is now under way.

It is conceivable that the salicylates may increase the resisting power of the animal body by stimulating the formation of immune bodies; but in so far as we are able to determine very little attention has been given to this possibility.

Jacoby and Schütze (2) studied the opsonin and bacteriotropin content of the serum of normal and immune rabbits; the serum was obtained both before and 8 hours after the animals received a single dose of 2 gm. of sodium salicylate by stomach tube. Staphylococci, *B. coli*, and *B. typhosus* were used in the tests. The authors state that five out of twelve rabbits showed a distinct increase of immune bodies following salicylate treatment. Unfortunately, the details of the experiments are not given, so it is impossible to tell whether the opsonins or the bacteriotropins were increased the more.

EXPERIMENTAL.

In view of the fact that no definite etiologic agent has been demonstrated for rheumatic fever, it was thought advisable in our studies to use various members of the coccus group of bacteria as antigens to stimulate the formation of antibodies in rabbits, and to compare the amount of antibody formation in immunized animals receiving sodium salicylate with that of animals similarly immunized but untreated with salicylates. In some of the experiments living bacteria were inoculated into the rabbits, in others killed bacteria in the form of vaccines were employed. In all instances the antigens were injected intravenously. In addition, three experiments were performed to determine the effect of salicylates upon hemolysin formation.

The rabbits received the sodium salicylate in the form of a 2.5 or 5 per cent solution twice daily by stomach tube. In the earlier experiments this was combined with an equal amount of sodium bicarbonate; but later it was found that the salicylate was well tolerated without the bicarbonate so the latter was omitted. In most instances the daily dose of sodium salicylate was from 0.16 to 0.2 gm. per kilo of body weight; the weight of the animals was determined from an average computed by repeated weighings for several days before the actual start of the experiments. Subsequent change in the weight of the rabbits was not used as an indication for altering the dose.

The rabbits were bled before beginning inoculation and twice a week subsequently. The separated serum was kept in the ice box, and the sera obtained at several different dates were tested at one time against the homologous antigens. This precaution was taken so that the results might be as comparable as possible. In all except the first two or three experiments a single standardized pipette was used to dilute all of the sera in one series of tests; this pipette was washed five or six times with saline solution after making each dilution. In doing comparative work with dilutions of different sera this precaution was necessary, for the ordinary pipettes were found to vary from 10 to 20 per cent. After recording the strength of reaction in the ordinary way by a series of plus marks the antibody content was computed from an interpolation table and curve. In this way the strength of reaction can be recorded by means of curves or figures and the comparison made easier than when the reactions are merely recorded by a certain number of pluses.

In some of the earlier experiments the temperature of the rabbits was charted twice daily; subsequently this procedure was discontinued as it was found that the information obtained was of little value in interpreting the immunity results.

Influence of Salicylates on the Formation of Complement-Fixing Antibodies.

In the first three experiments the formation of complement-fixing antibodies against the homologous streptococci was followed.

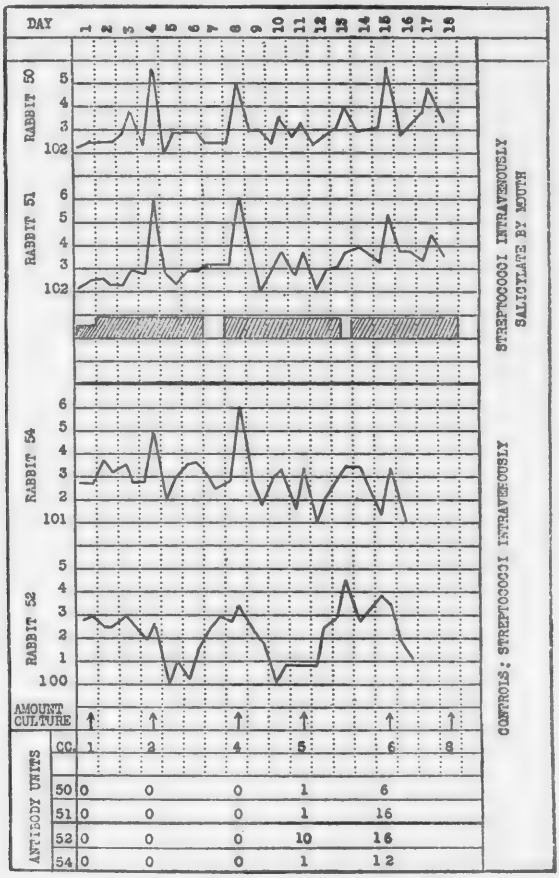
The antigens used in the tests were prepared by dissolving dried bacteria in antiformin and titrating this solution back to neutral according to the method described by Kinsella and Swift (3). The reactions were carried out in the usual manner, with a period of 1 hour for incubation of antigen, complement, and antibody; after which sensitized sheep red blood cells were added and the tubes returned to the water bath at 37°C. for 2 hours. Immediate readings were then made. The antigens were used in one-half the anticomplementary dose, and both the complement and hemolytic amboceptor in double the hemolytic dose.

Experiment 1.—Four rabbits of practically equal weight were chosen and weighed daily for a week; their temperatures were taken morning and evening as a control of the subsequent period. The animals were divided into two groups of two each. All received intravenous injections of living cultures of *Streptococcus viridans* A49 twice a week in amounts shown in Text-fig. 1. Rabbits 50 and 51 received daily sodium salicylate, 0.16 gm. per kilo of body weight. All of the sera were tested for complement-fixing antibodies on the same day with the same complement and antigen. The general results of the experiment are shown in Text-fig. 1.

It is evident that prolonged administration of sodium salicylate by stomach tube in the dose used had no deleterious effect on the rabbits. A loss of weight in all of the animals can be attributed to other factors than salicylates, such as the repeated inoculation with streptococci, or snuffles. Two control rabbits receiving the same amount of sodium salicylate, but not inoculated with streptococci, showed no loss in weight and little variation in the temperature curve.

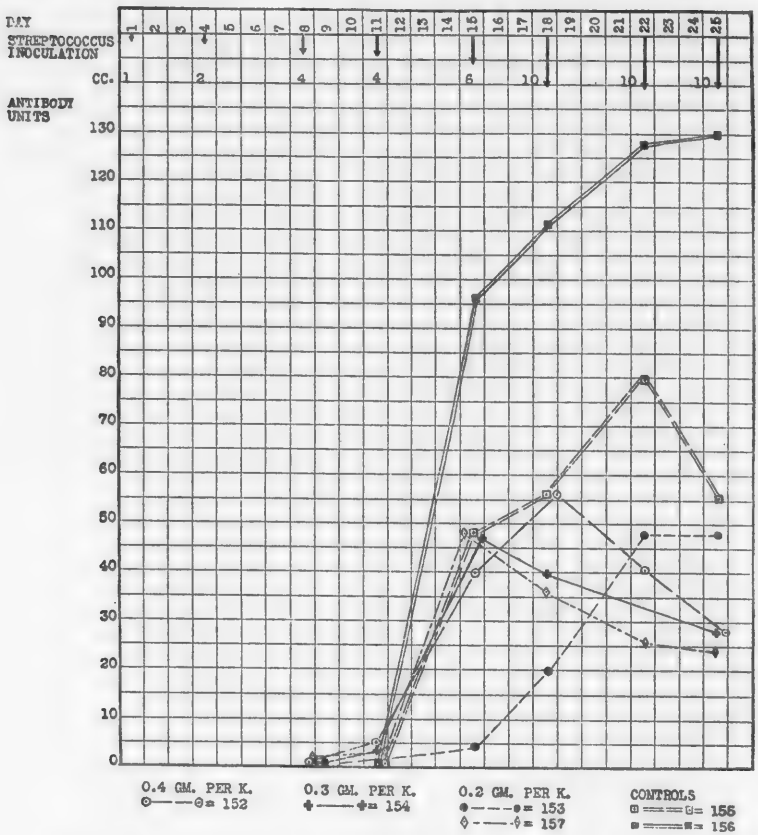
Apparently, the salicylates had little or no antipyretic effect, for the rabbits, whether receiving the drug or not, showed a similar fever within a few hours after intravenous inoculation of living streptococci. Rabbit 52 showed less effect following inoculation than did any of the others. The complement-fixing antibody is indicated in units. The first antibodies were detected in all of the rabbits on the 11th day following the first inoculation, when the two salicylated animals and one control showed equal titers; the other con-

trol had more antibodies. On the 15th day the combined non-salicylated controls yielded sera with stronger titer than did the salicylated animals. The experiment was terminated at this point because of the death of one of the controls.


DAY
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
RABBIT 50
RABBIT 51
RABBIT 54
RABBIT 52
STREPTOCOCCI INTRAVENOUSLY
SALICYLATE BY MOUTH
CONTROLS: STREPTOCOCCI INTRAVENOUSLY
AMOUNT CULTURE
ANTIBODY UNITS
CC. 1 2 4 5 6 8
50 0 0 0 1 6
51 0 0 0 1 16
52 0 0 0 10 16
54 0 0 0 1 12

TEXT-FIG. 1. Comparison of temperature curves and complement-binding antibody units in salicylated and control rabbits inoculated twice weekly with living *Streptococcus viridans* A49. The cross-hatching indicates periods of sodium salicylate administration.

Experiment 2.—Of six rabbits, two served as controls; the other four received sodium salicylate by stomach tube, two doses a day, as follows: No. 152, 0.4 gm. per kilo of body weight a day; No. 154, 0.3 gm.; Nos. 153 and 157, 0.2 gm. All of the animals were inoculated twice a week with increasing amounts of living



TEXT-FIG. 2. Comparison of complement-binding antibodies in rabbits receiving different amounts of sodium salicylate with controls; animals immunized twice weekly with living *Streptococcus viridans* A49.

Streptococcus viridans A49, as indicated in Text-fig. 2. Blood was obtained before each inoculation; the separated sera were stored until the termination of the experiment, and all were tested simultaneously against the homologous streptococcus for complement-fixing antibodies. The experiment was terminated after the 25th day; the results are shown in Text-fig. 2.

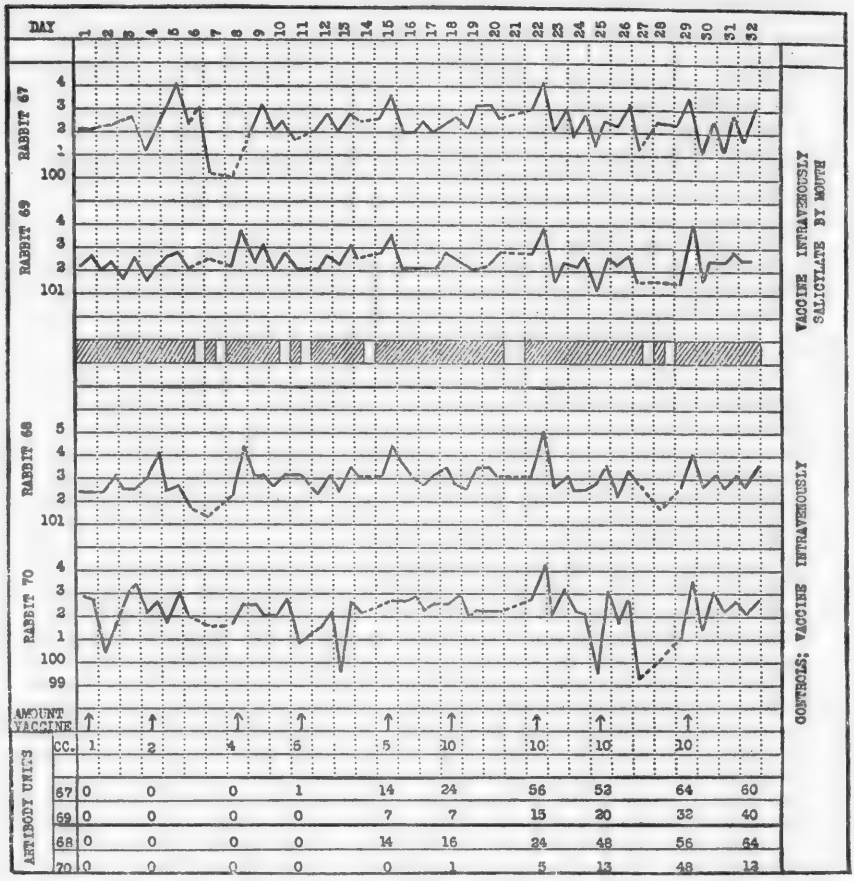
It is evident that there is no striking difference in the antibody content of the rabbits that received different amounts of sodium salicylate; the variation is no greater than might be seen in four different untreated rabbits. It is noteworthy that the two non-salicylated controls showed higher antibody curves than did any of the salicyl-treated animals.

All of the animals were weighed, and all showed similar losses of from 100 to 200 gm.; therefore, in so far as this feature is concerned, neither deleterious nor beneficial effects could be attributed to the salicylates.

Experiment 3.—The same general plan was followed as in Experiment 1, with the following exception. The rabbits were immunized with a stock vaccine prepared by suspending in normal saline solution the centrifugate of a 24 hour broth culture of hemolytic Streptococcus K, and killing the bacteria by heating at 56°C. for ½ hour. The animals were immunized by injecting intravenously increasing amounts of this vaccine. Temperature and weight curves of the rabbits were followed; only the temperature curves are reproduced, as the weights of three of the animals increased during the experiment, and that of the fourth was stationary. Blood was withdrawn before each inoculation; the sera were tested at two different periods, after the 18th and 32nd days, respectively. The number of complement-fixing units is recorded in Text-fig. 3.

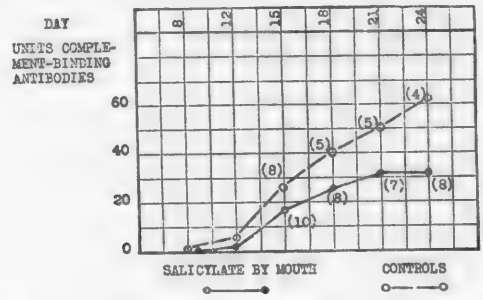
It is evident that there was little if any difference in the antibody content of the two groups of animals; the difference is no greater than might be expected in such small groups. The fever normally following the intravenous injections of bacterial vaccines was uninfluenced by the administration of salicylates in the same manner as was the fever following the intravenous inoculation of living *Streptococcus viridans*.

A similar experiment was conducted in which the rabbits were inoculated with living cultures of hemolytic Streptococcus K. These rabbits died from generalized streptococcus infection before sufficient time had elapsed to obtain comparable results. Here, again, no distinct antipyretic effect was seen in the salicyl-treated animals as compared with untreated controls. All four of the rabbits inoculated with living hemolytic streptococci had multiple purulent arthritis from which pure cultures of hemolytic streptococci were recovered. It is therefore evident that the salicylates had no influence in preventing the development of arthritis.



TEXT-FIG. 3. Comparison of temperature curves and complement-binding units in salicylated and control rabbits immunized twice weekly with vaccines of *Streptococcus hæmolyticus* K. The cross-hatching indicates periods of sodium salicylate administration.

A summary of all of the experiments showing the influence of salicylates upon the formation of complement-fixing antibodies is given in Text-fig. 4. Here the averages derived from a larger group of animals than was used in any individual experiment indicates that salicylates in general had a depressing influence upon the formation of this type of immune bodies.



TEXT-FIG. 4. Comparison of average complement-binding antibody formation in the foregoing series of salicylated and control rabbits. The number of animals tested each day is indicated in parenthesis.

Influence of Salicylates on the Formation of Agglutinins.

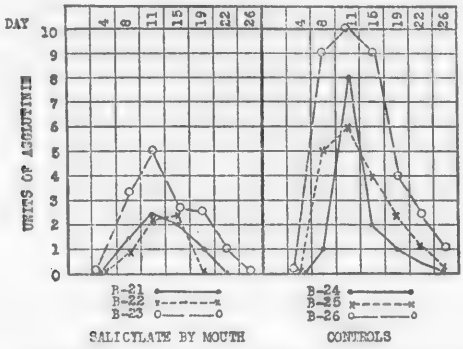
In the next four experiments the rate of agglutinin formation was determined.

In all experiments the sera were tested against killed cultures of the homologous bacteria prepared as follows: A 24 hour broth culture was heated at 56°C. ½ hour, and standardized by Gates' (4) method so that the density of the suspensions was similar. The reaction of the suspensions was brought to a pH 7.3 or 7.4 to avoid acid agglutination. In order to insure that the suspending medium was the same in all of the tubes the sera were diluted with broth, instead of salt solution. The tubes were incubated at 56°C. for 2 hours, removed from the incubator, and allowed to cool for 15 minutes; then the strength of reaction was recorded.

Experiment 4.—Six brown rabbits weighing from 1,400 to 1,600 gm. were immunized with a pneumococcus of Type I by giving daily intravenous injections of vaccines prepared by suspending the centrifugate of 24 hour broth cultures in normal saline solution and heating at 56°C. for ½ hour. An amount of vaccine representing 2 cc. of culture was given to each rabbit daily for 6 days. Three of the rabbits (Nos. B-21, B-22, and B-23) received sodium salicylate daily for

14 days; the dose was 0.16 gm. per kilo of body weight. The other three rabbits served as controls. The sera of all of the animals were tested for agglutinin content against the same suspension of homologous pneumococci. The results are given in Text-fig. 5.

In this experiment the amount of immunizing antigen was small and the period of immunization was short in comparison with the previous experiments. It is difficult to obtain in rabbits an agglutinating serum of high titer against pneumococci. In this series, therefore, the curves are low; the salicylated rabbits, nevertheless, had uniformly less antibodies than did the controls.



TEXT-FIG. 5. Comparison of agglutinin formation in salicylated and control rabbits immunized with six daily injections of pneumococcus vaccine.

Experiment 5.—Ten brown rabbits weighing between 1,700 and 1,800 gm. each were divided into two lots of five. One lot was given sodium salicylate, 0.16 gm. per kilo of body weight, daily for 20 days. On the 2nd day all of the animals received intravenously the centrifugalized sediment of 5 cc. of broth culture of *Streptococcus viridans* 38D; on the 6th day a similar amount of the same organism; and on the 14th day 10 cc. Blood was obtained from the animals before the beginning of treatment and on the 6th, 10th, 14th, 17th, and 21st days. Three of the salicylate-treated animals and one of the control group died during the course of the experiment; all of the surviving animals were killed and examined for lesions of the joints and viscera on the 20th or 21st day. At the termination of the experiment all of the sera were titrated for agglutinins. The final interpolated titers of the various sera are given in Table I.

TABLE I.

Comparison of Agglutinin Formation in Salicylated and Control Rabbits Inoculated with Living Streptococcus viridans 38D.

Date.	Day.	Inoculation.	Salicylated rabbits.							Controls.						
			A-52	A-54	A-56	A-58	A-60	A-62	Average.	A-53	A-55	A-57	A-59	A-61	A-65	Average.
1921																
Apr. 2			0	0	0	0	0	0		0	0	0	0	0	0	
" 7	2	5 cc. of Streptococcus 38D.														
" 11	6		10	8	10	20	18	18	14	27	8	18	16	20	17	18
" 11	6	5 cc. of Streptococcus 38D.														
" 15	10		18	10	30	120	96	64	56	76	20	80	96	70	76	69
" 18	10	10 cc. of Streptococcus 38D.														
" 19	14		224	176	256	480	320	Dead.	291	480	80	480	1, 120	224	880	544
" 22	17		510	304	Dead.	512	560	"	471	640	152	512	1, 200	Dead.	1, 280	757
" 26	21		510	640	"	1, 200	Dead.	"	783	350	1, 040	880	2, 240	"	2, 400	1, 382

Although the usual variation in the agglutination titer in the serum of different animals is seen in both groups, most of the rabbits in the control group consistently yielded stronger agglutinating serum than did the salicyl-treated rabbits; the average curve of the control group was uniformly higher than that of the salicyl group, and on the 14th, 17th, and 21st days it was nearly twice as high.

In another experiment carried out in a manner similar to No. 5, part of the salicylated rabbits succumbed to intravenous inoculations of Streptococcus A49; on the 8th day the three surviving rabbits in this group gave an average agglutinin content of 46 units; and six controls gave an average content of 57 units. On the 16th day only a single salicylated animal was living, while five controls still survived; at this time both groups had an average agglutinin content of 900. Because of the difference in the number of animals in the two groups this experiment is not given in detail. It merely shows that a single salicylated animal may have as high an antibody curve as some untreated controls; and emphasizes the desirability of having more than one animal in each group.

In still another experiment in which only two salicylated rabbits and four controls lived until the 6th day, the average agglutinin curve on this day was 735 for the first and 450 for the second group. These animals were immunized with a single intravenous injection of 35 cc. of *Streptococcus viridans* A135. The difference in the number of animals in the two groups also decreases the value of the results in this experiment. It is possible that the large amount of immunizing antigen may have been a factor in eliminating the difference in the two groups of animals.

These two experiments were the only ones in which the animals receiving salicylates yielded serum with as high or higher average antibody content than that of control animals.

Experiment 6.—This experiment was designed to determine whether salicylate-treated rabbits and non-salicylated controls, all previously inoculated into the knee joints with killed cultures of green streptococci, would show any difference in the agglutinating power of their sera after intravenous inoculation with living streptococci.

Each of six brown rabbits weighing between 1,600 and 1,900 gm. was inoculated into the right knee with the sediment of 0.5 cc. of broth culture of *Streptococcus viridans* 38D killed by heating for ½ hour at 56°C. and into the left knee with 0.5 cc. of *Streptococcus viridans* A49 similarly treated. The knees were measured and otherwise observed until they had reached their original dimensions or were stationary in size. Serum was separated from blood obtained on the 3rd, 20th, and 28th days following intraarticular inoculation. The animals were then divided into two groups of three each, and the members of one group given

sodium salicylate daily in doses of 0.2 gm. per kilo of body weight; this treatment was started on the 27th day after the intraarticular inoculation. The next day each of the animals received an intravenous injection of the sediment of living streptococci from 9.3 cc. of an 18 hour culture of Streptococcus 38D. Serum was obtained on the 3rd and 9th days following this intravenous inoculation, and on the 9th day all of the animals were autopsied to determine the extent of articular and visceral lesions.

In the immunity studies several points may be noted: (1) the possibility of detecting agglutinins in the blood following the intraarticular inoculation of small amounts of two different strains of killed streptococci; (2) the effect of subsequent intravenous inoculation of one of the two strains—whether it stimulates antibodies to one or both; and (3) the difference in the salicylate-treated and control groups. The results are summarized in Table II.

Although the antibody curve was not followed at frequent intervals after the intraarticular injection of killed streptococci, it is evident that a single injection of Strain A49 stimulated the formation of agglutinins much more than did a simultaneous injection of Strain 38D. It is impossible to state whether the agglutinins detected on the 28th day were stimulated by the intraarticular injection or by the intravenous inoculation because the blood was withdrawn 8 hours after the intravenous inoculation. The increase in agglutinins for Strain A49 on the 9th day after the intravenous injection of Strain 38D must have been due to the stimulating effect of the latter strain in animals already slightly immune to No. A49. The rise in antibody curve at this time was noted for both strains. The two strains are distinct antigenically; the serum of many animals immunized alone with Strain 38D has never agglutinated No. A49, and *vice versa*. The increase in antibodies for No. A49 on the last day must, therefore, have been a concomitant phenomenon, and did not seem to have been influenced by salicylates. The lower agglutinin content for Streptococcus 38D in the salicylated animals is similar to that observed in other experiments.

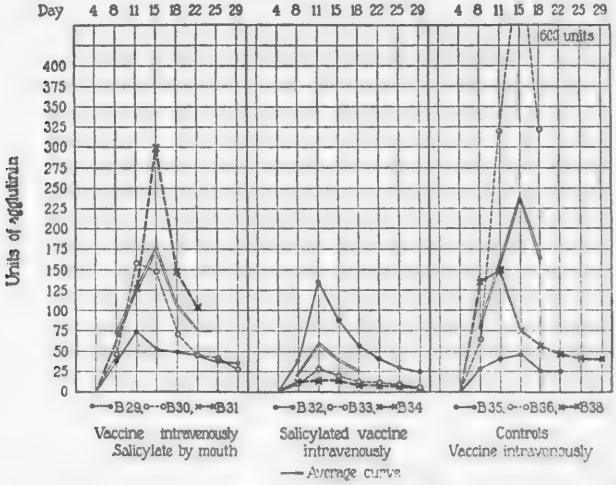
Experiment 7.—The object of this experiment was to determine in addition whether streptococci treated *in vitro* with salicylates were less active antigenically than untreated streptococci. Three groups of animals were chosen. Group 1, consisting of Rabbits B-29, B-30, and B-31, received sodium salicylate by stomach

TABLE II.

Comparison of Agglutinin Formation in Salicylated and Control Rabbits Inoculated with Streptococcus viridans 38D, but Previously Injected into the Knee Joints with Streptococcus viridans 38D and A49.

Date.	Day.	Inoculation.	Agglutination tested with Streptococcus 38D.								Agglutination tested with Streptococcus A49.					
			Salicylated rabbits.				Controls.				Salicylated rabbits.			Controls.		
			K-23	K-25	K-28	Average.	K-22	K-24	K-26	Average.	K-23	K-25	K-28	K-22	K-24	K-26
1921																
Apr. 21	1	Right knee 0.5 cc. of killed Streptococcus 38D. Left knee 0.5 cc. of killed Streptococcus A49.														
" 23	3		0	0	0		0	0	0		0	0	0	0	0	0
May 10	20		0	0	1		0	0	1		1	0	0	2	1	1(?)
" 18	28		0	0	0		0	0	0		2	2	0	0	1	0
" 18	1	9.3 cc. of living Streptococcus 38D intravenously.														
" 20	3		0	0	0		0	0	1		2	0	1(?)	0	1(?)	0
" 26	9		480	140	480	366	300	720	560	526	8	2	4	2	4	4

tube, 0.2 gm. per kilo of body weight, and daily injections of vaccine prepared from broth cultures of *Streptococcus viridans* 38D. The vaccine was given for 5 days. Group 2, consisting of Rabbits B-32, B-33, and B-34, received daily intravenous injections of salicylated vaccine. This vaccine was prepared as follows: An aliquot portion of the vaccine used in immunizing Group 1 was mixed with an amount of sodium salicylate solution that would have been used had the rabbit received the drug by stomach tube. The vaccine and salicylate were incubated at 37°C. for 4 hours, then centrifugalized, and the centrifugate was taken up in saline solution and injected intravenously. This vaccine was given for 5 days. Group 3, the control group, consisting of Rabbits B-35, B-36, and B-38, received



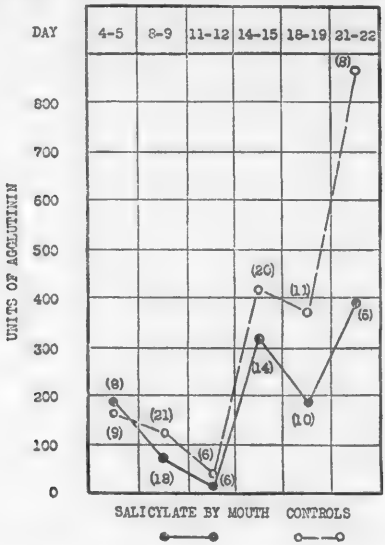
TEXT-FIG. 6. Comparison of agglutinin formation in salicylated and control rabbits immunized with vaccines of *Streptococcus viridans* 38D, and rabbits immunized with salicylated vaccines.

daily injections of untreated vaccine. All of the rabbits received each day vaccines in an amount equal to 2 cc. of broth culture per kilo of body weight. The rabbits were bled before the experiment and on other days indicated in Text-fig. 6. All of the sera were tested for agglutinin content on the same day. The results are shown in Text-fig. 6.

Here, as in previous experiments, the control group had a higher agglutinin content in their sera than did the rabbits treated with sodium salicylate. The second group, namely those that received salicylated vaccine, had the lowest antibody content. In this experi-

ment it seemed, therefore, that the action of the salicylate might be directly upon the antigen, causing it to be a less active stimulator of antibody production in the animal body.

It was thought advisable to determine whether streptococci treated with salicylate might act differently towards an agglutinating anti-



TEXT-FIG. 7. Comparison of average agglutinin formation in the complete series of salicylated and control rabbits. The number of animals tested each day is indicated in parenthesis.

serum than untreated streptococci. The following experiment was therefore performed.

A 24 hour broth culture of Streptococcus 38D was killed by heating ½ hour to 56°C. The reaction of the medium at this time was pH 7.4. The culture was divided into two parts: X was kept in the original state; Y was treated as follows: It was centrifugalized and the supernatant serum broth retained. The separated bacterial sediment was mixed with 400 cc. of 5 per cent sodium salicylate and incubated for 4 hours at 37°C. It was then centrifugalized to clearness, the supernatant sodium salicylate solution discarded, and the bacterial sediment added to the original broth. The resuspension of the cocci in the medium in which they were grown insures that Suspensions X and Y were the same except that Suspension Y contained salicylated streptococci. These two suspensions were

then tested against the sera of Rabbits B-29, B-31, B-32, B-33, and B-34. With each serum the agglutination reached the same point in both suspensions; the differences in the agglutinating power of the serum of the rabbits of Groups 1 and 2 cannot, therefore, be attributed to the possibility that Group 2 rabbits were immunized with less agglutinable streptococci.

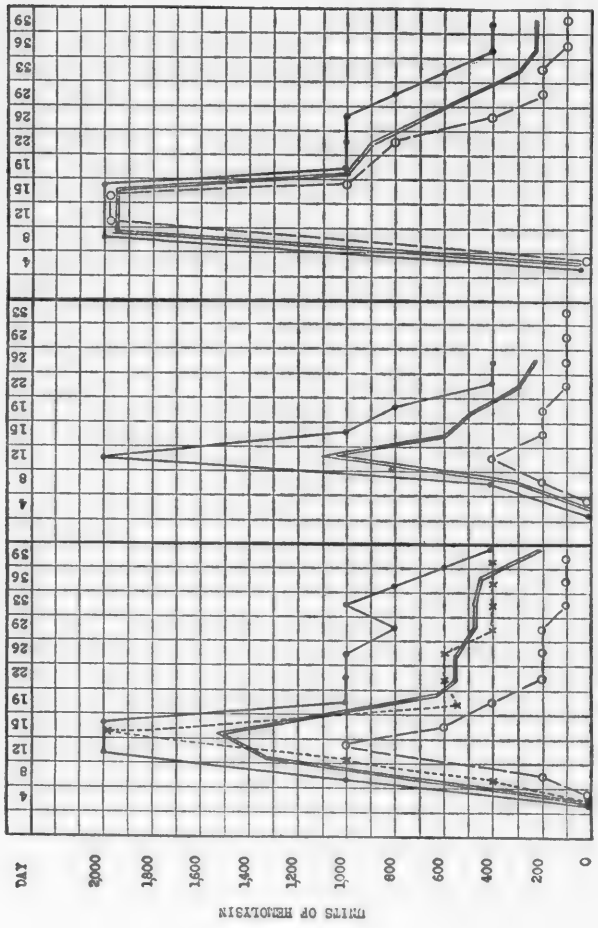
The average agglutinin content of the serum of all of the rabbits used in the foregoing experiments is shown in Text-fig. 7. The irregular course of the curves is due to the difference in the number of animals tested at different periods. At each period, however, the number of controls is equal to or greater than the number of salicylated animals. This summary shows that the average agglutinin formation in the entire group of salicylated animals was lower than that of the controls, and is in accord with the findings of the animals tested for complement-binding antibodies.

Influence of Salicylates on the Formation of Hemolysin.

In the remaining experiments the antibody tested was an hemolysin against sheep red blood cells.

All of the sera were inactivated by heating at 56°C. for ½ hour. The diluted serum was then mixed with guinea pig serum so diluted that 0.5 cc. equaled one unit of complement previously tested with a constant standard hemolytic serum. To the rabbit serum and guinea pig complement was added 0.5 cc. of a 5 per cent suspension of sheep red blood cells. The mixtures were incubated in the water bath at 37°C. for 2 hours and read immediately. The dilution at which hemolysis was complete was considered as containing the charted unit of antibody. Because of the result obtained in Experiment 7 the experiments with hemolysins were all carried out with three groups of animals: Group 1, in which the animals received washed sheep red blood cells intravenously and sodium salicylate by stomach tube; Group 2, in which the animals received intravenous injections of salicylated sheep red blood cells, prepared by incubating together washed erythrocytes and a 2.5 per cent sodium salicylate solution, then centrifugalizing the mixture and retaining the cells alone for inoculation; Group 3, in which the animals received simply intravenous injections of washed sheep red blood cells.

Experiment 8.—Seven rabbits about 6 weeks old, all brown, of the same litter, were chosen for this experiment. Inactivated serum obtained from a preliminary bleeding was tested for hemolysin content against sheep red blood cells. The rabbits were then divided into three groups according to their weight and the native hemolysin content of their serum. This precaution was taken because certain investigators claim that the amount of antibody formation following



TEXT-FIG. 8. Comparison of hemolysin formation in salicylated and control rabbits immunized with sheep erythrocytes, and rabbits immunized with salicylated erythrocytes Salicylate and erythrocytes given daily for 3 days, 1 day intermission, then for 3 more days.

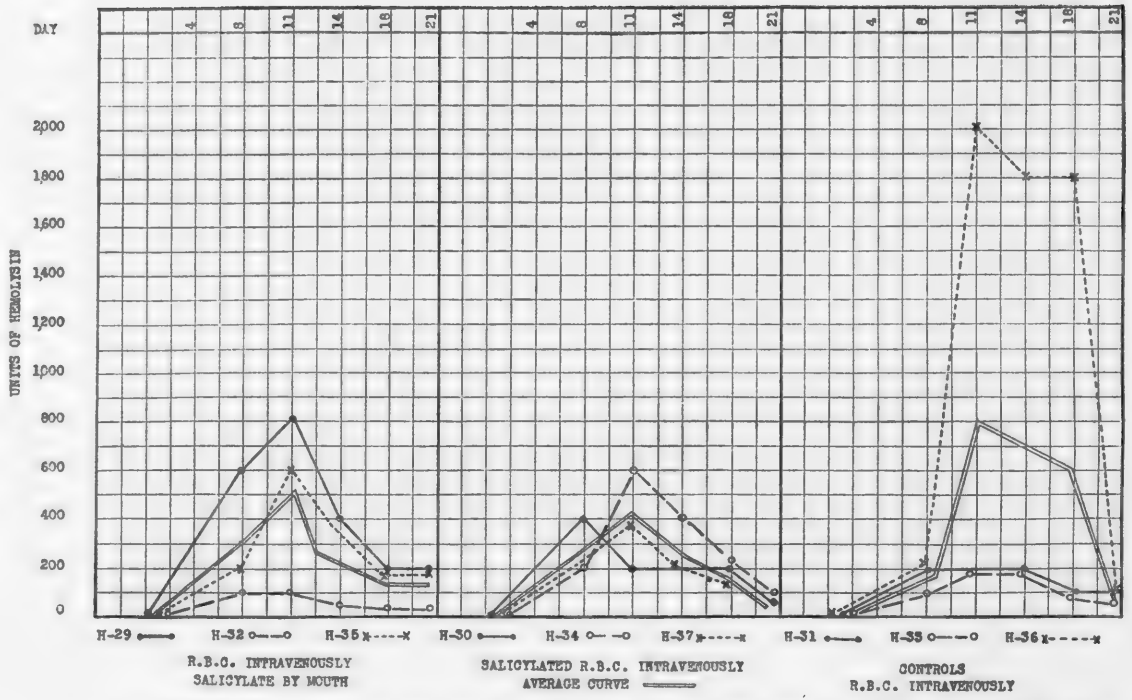
immunization is more or less in direct proportion to the amount of antibody the serum of the animal contained before immunization. Group 1, consisting of Rabbits B-43, B-45, and B-46, received injections of sheep cells daily for 3 days followed by an interval of 1 day, and then for 3 more days, and at the same time sodium salicylate by stomach tube—a total daily amount of 0.16 gm. per kilo of body weight per day. Group 2, consisting of Rabbits B-44 and B-49, received intravenous injections on the same days as did rabbits of Group 1. They received sheep red blood cells that had been incubated for 3 hours with sodium salicylate, the supernatant salicylate solution discarded, and the salicylated cells resuspended in saline solution. Group 3, controls, consisting of Rabbits B-47 and B-48, was injected with washed sheep blood cells. The antibody curves of these rabbits are shown in Text-fig. 8.

In all three groups some of the rabbits showed hemolysin titers as high as 1,000 to 2,000. Comparing the average curve for each group, however, the highest and most prolonged antibody content was found in Group 3, the next in Group 1, and the lowest in Group 2, the group immunized with salicylated cells.

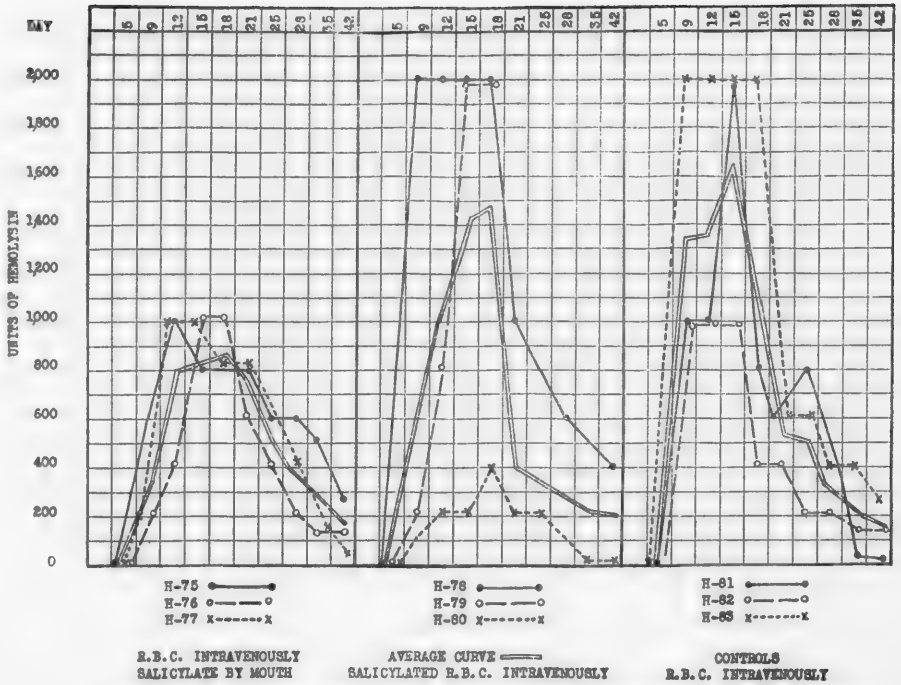
Experiment 9.—This experiment was carried out in essentially the same manner as Experiment 8, with the following exceptions. Three groups of larger rabbits, consisting of three animals each, were used; injections of erythrocytes were given only 3 days; and the sodium salicylate by mouth was given for a similar period. The influence of the salicylate was, therefore, correspondingly less than in animals receiving the drug over a longer period. The results are shown in Text-fig. 9.

In this experiment the differences between the various groups were less marked than in previous experiments although the average curve in the control group (Group 3) was higher than in Groups 1 and 2. This high average is due to the fact that the serum of Rabbit H-36 had a much higher antibody content than that of any of the others. The other two rabbits in this group had a lower antibody content than any of the rabbits in the other two groups. As in Experiment 8 the animals receiving salicylate by stomach tube had a higher average curve than did those receiving salicylated red blood cells intravenously.

Experiment 10.—A comparison of the results in Experiments 8 and 9 seems to indicate that a long or short period of administration of the salicylates might be a factor in determining the degree of antibody formation. Experiment 10 was made to test the influence of more prolonged administration of the drug. The rabbits used in this experiment were all brown, full grown, and of approximately

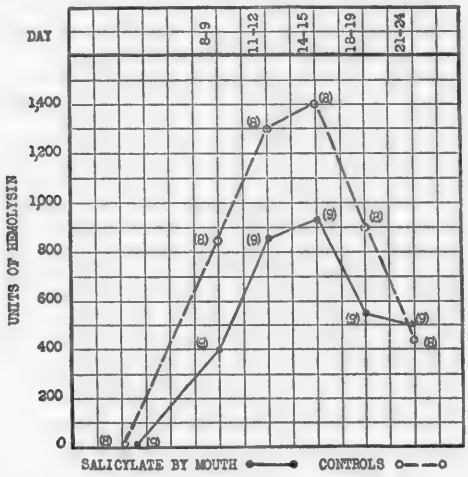


TEXT-FIG. 9. Comparison of hemolysin formation in salicylated and control rabbits immunized with sheep erythrocytes, and rabbits immunized with salicylated erythrocytes. Salicylate and erythrocytes given daily for 3 days.



TEXT-FIG. 10. Comparison of hemolysin formation in salicylated and control rabbits immunized with sheep erythrocytes and rabbits immunized with salicylated erythrocytes. Salicylate given for 12 days; erythrocytes injected on the 1st, 5th, and 9th days.

the same size. Three intravenous injections of 25 per cent sheep red blood cells, 4 cc. per kilo of body weight, were given to each rabbit on the 1st, 5th, and 9th days, respectively. In Groups 1 and 3 the cells were not salicylated; in Group 2 the cells were salicylated by incubating them with 2.5 per cent sodium salicylate solution for 15 hours, the suspension centrifugalized, the supernatant solution discarded, and the cells resuspended in saline solution. Group 1 animals received sodium salicylate in a total daily amount of 0.2 gm. per kilo of body weight. The animals received three doses before the first immunizing cells were given and were treated 4 days after the last intravenous injection of erythrocytes. The results are shown in Text-fig. 10.



TEXT-FIG. 11. Comparison of average hemolysin formation in the salicylated and control rabbits used in Experiments 8 to 10. The number of rabbits tested each day is indicated in parenthesis.

Here, as in previous experiments, the control group had a higher total as well as higher average antibody content than did the other two groups. Group 1, in which the rabbits received salicylate for 12 days, showed the lowest antibody content. Group 2, treated with salicylated cells, showed almost as great an antibody content as did Group 3. The discrepancy between the animals in Group 2 in this experiment and in the three previous experiments cannot be explained.

If the average hemolysin content of the sera of all of the animals used in Experiments 8 to 10 are plotted (Text-fig. 11), it is seen that

the control animals not receiving salicylate yielded sera with consistently higher titer than did animals receiving salicylate by mouth. This is in complete accord with the results obtained for agglutinating and complement-binding antibodies.

DISCUSSION.

Aside from the two incomplete experiments in which the number of animals in the salicylated group was small, the controls all had a higher antibody content than did animals similarly immunized but treated with sodium salicylate. The dose of salicylate given the rabbits each day was comparable with the largest amount that may be safely administered to patients for short periods, and much larger than can be tolerated over a long period by patients with rheumatic fever. While some rabbits receiving 0.4 gm. per kilo each day seemed to suffer no deleterious effects, others receiving this amount of the drug succumbed more quickly to intravenous inoculation of living streptococci than did unsalicylated controls.

Fantus, Simmonds, and Moore (5) showed that sodium salicylate, when used in a dose which they state was comparatively harmless to animal controls, is decidedly detrimental and liable to be fatal to rabbits infected with hemolytic streptococci. In their experiments from 0.9 to 1 gm. of sodium salicylate and 1.2 gm. of acetylsalicylic acid were given by mouth each day; in another series 0.3 gm. of sodium salicylate was given hypodermically. These are much larger doses than may be given with safety to patients. Our experiments indicate that 0.2 gm. is as large a daily dose as can be safely administered to rabbits over long periods.

Rabbits receiving the drug daily by stomach tube at first refused to take much food, but after 3 or 4 days they again resumed their normal feeding habits. Malnutrition in this group of animals cannot, therefore, be considered as the cause of a lowered antibody formation.

It is possible that animals with less febrile response following the intravenous injection of antigens might show a correspondingly lower antibody formation. As the salicylates have a marked antipyretic effect in patients this argument might be used theoretically to explain our results. In the first and third experiments it was shown, however, that salicylates in the doses employed did not have an anti-

pyretic effect in inoculated rabbits. A hypothetical correlation between fever and antibody formation must, therefore, be abandoned in explaining the results.

Only a few drugs or chemicals have been tested to determine their action upon the formation of immune bodies.

With the introduction of salvarsan several workers (6, 7) claimed that one of the marked actions of arsenicals in general was to increase antibodies. Others (8, 9) have claimed that this effect was less marked and less constant than found by the earlier experimenters. Toyama and Kolmer (9) state that large doses of both salvarsan and mercury bichloride appear to depress hemolysin and agglutinin production in immunized rabbits; while smaller doses of both drugs tend to increase the production of hemagglutinins, but not of hemolysins.

Müller (10) found that animals treated with aleuronat or cinnamic acid while undergoing immunization yielded more antibodies than did controls. He thought that because these two substances stimulated leucocytosis the two effects were to be attributed to a common action. Similarly the depression in immune bodies in animals under the influence of benzene led Hektoen (11) to believe that substances exerting a destructive action on the blood-forming organs had a depressing effect on the tissues from which immune bodies arise. But his later studies concerning the effect of toluene (12) and thorium (13) on immune body production indicate that variations in the antibody content of the blood serum and leucocytosis or leucopenia need not necessarily go hand in hand.

Abbott and Bergey (14), Müller (10), and Friedberger (15) have all demonstrated conclusively that alcohol in repeated doses has a very depressing influence on the capacity of immunized animals to form antibodies. Melnikowa and Wersilowa (16), and Müller (10) have shown that phlorhizin has a similar effect. Hektoen and Corper (17) have shown that mustard gas (dichloroethylsulfide) depresses immune body formation.

The foregoing review of previous experiments fails to furnish any explanation that can be brought to bear upon our studies.

Cook (18) has shown that the rate of antibody formation is dependent upon the rate of absorption of the antigen. She reviews and makes use of the work of Loeb, Lillie, and Osterhout, all of whom have demonstrated that the permeability of cells is increased by increasing the sodium ions and decreased by increasing the calcium ions. In her experiments, rabbits treated with sodium citrate formed antibodies more intensely than did controls, and those treated with calcium chloride less intensely than controls. In all of our experiments the rabbits receiving salicylates were at the same time subject to an

increased amount of sodium from the sodium salicylate. From Cook's experiments one would expect an increased antibody output if the sodium ion is the important element in the compound. Whether the salicyl ion has an antagonistic effect by decreasing the absorption of antigen is a question that must be submitted to experimental study. Reasoning simply by similarity of effect it seems possible that the decreased antibody production in rabbits treated with sodium salicylate may be due to the decreased power of these animals to absorb antigen.

It must be emphasized in this connection that there is nothing in our experimental results to indicate that the salicylates are deleterious in infection simply because they decrease immune body formation. In none of the experiments was this decrease extremely marked, and all of the salicylated animals produced immune bodies in fair amounts. It is probable that immune bodies in the circulating fluids are not the only factors concerned in the process of recovery. The same mechanism that makes an infectious agent less antigenic might at the same time make it less pathogenic. It is possible that this effect may be more marked in rheumatic fever than in other infections; hence the more striking effect of the drug seen in this disease.

CONCLUSION.

1. Rabbits treated with sodium salicylate in daily doses of from 0.16 to 0.2 gm. per kilo of body weight and at the same time immunized with intravenous injections of *Streptococcus viridans*, both living and in the form of vaccines, and also with washed sheep red blood cells, showed diminished complement-fixing antibodies, agglutinins, and hemolysins when compared with controls similarly immunized.

2. If the antigens were treated with sodium salicylate ***in vitro*** and subsequently injected intravenously into rabbits, the animals usually showed lower antibody curves than did rabbits that received the untreated antigen intravenously and sodium salicylate by stomach tube.

3. The beneficial effect of sodium salicylate in rheumatic fever patients probably cannot be attributed to an increased production

of circulating immune bodies against the infectious agent. This is, however, no contraindication to the administration of salicylates to patients suffering from infectious diseases.

BIBLIOGRAPHY.

1. Barbour, H. G., *Arch. Int. Med.*, 1919, xxiv, 624. Barbour, H. G., and Devenie, M. M., *Arch. Int. Med.*, 1919, xxiv, 617.
2. Jacoby, M., and Schütze, A., *Biochem. Z.*, 1908, ix, 527.
3. Kinsella, R. A., and Swift, H. F., *J. Exp. Med.*, 1917, xxv, 877.
4. Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.
5. Fantus, B., Simmonds, W. E., and Moore, J. J., *Arch. Int. Med.*, 1917, xix, 529.
6. Friedberger, E., and Masuda, N., *Therap. Monatsh.*, 1911, xxv, 288.
7. Boehncke, K. E., *Z. Chemotherap., Orig.*, 1912–13, i, 136.
8. Reiter, H., *Z. Immunitätsforsch., Orig.*, 1912, xv, 116.
9. Toyama, I., and Kolmer, J. A., *J. Immunol.*, 1918, iii, 301.
10. Müller, P. T., *Arch. Hyg.*, 1904, li, 365.
11. Hektoen, L., *J. Infect. Dis.*, 1916, xix, 69.
12. Hektoen, L., *J. Infect. Dis.*, 1916, xix, 737.
13. Hektoen, L., and Corper, H. J., *J. Infect. Dis.*, 1920, xxvi, 330.
14. Abbott, A. C., and Bergey, D. H., *Centr. Bakt., Ite Abt., Orig.*, 1902, xxxii, 260.
15. Friedberger, E., *Berl. klin. Woch.*, 1904, xli, 242.
16. Melnikowa, F. J., and Wersilowa, M. A., *Centr. Bakt., Ite Abt., Orig.*, 1912, lxvi, 525.
17. Hektoen, L., and Corper, H. J., *J. Infect. Dis.*, 1921, xxviii, 279.
18. Cook, M. W., *J. Immunol.*, 1920, v, 39.

INDEX TO AUTHORS.

A

Amoss, Harold L. Experimental epidemiology. I. An artificially induced epidemic of mouse typhoid, 25

—. II. Effect of the addition of healthy mice to a population suffering from mouse typhoid, 45

— and **Haselbauer, Peter P.** Immunological distinctions of two strains of the mouse typhoid group isolated during two spontaneous outbreaks among the same stock, 107

D'Aunoy, Rigney. See DUVAL and D'AUNOY, 231, 239

B

Bauer, Johannes H. See TEN-BROECK and BAUER, 261

Bien, Z. See STORM VAN LEEUWEN, BIEN, and VAREKAMP, 415

Bliss, Walter Parks. Studies on the biology of streptococcus. II. Antigenic relationships between strains of *Streptococcus hæmolyticus* isolated from scarlet fever, 575

Boots, Ralph H., and Cullen, Glenn E. The hydrogen ion concentration of joint exudates in rheumatic fever and other forms of arthritis, 405

Broun, G. O. Blood destruction during exercise. I. Blood changes occurring in the course of a single day of exercise, 481

Brown, Thomas K. See LEAKE and BROWN, 661

C

Carrel, Alexis. Growth-promoting function of leucocytes, 385

— and **Ebeling, Albert H.** Action of shaken serum on homologous fibroblasts, 399

— and —. Leucocytic secretions, 645

— and —. Pure cultures of large mononuclear leucocytes, 365

Cowdry, Edmund V. The supravital staining of vaccine bodies, 667

— and **Olitsky, Peter K.** Differences between mitochondria and bacteria, 521

Craighead, Eugene M. See WRIGHT and CRAIGHEAD, 135

Cullen, Glenn E. See BOOTS and CULLEN, 405

D

De Kruif, Paul H. Rabbit septicemia bacillus, Types D and G, in normal rabbits, 309

Dougherty, Katharine M. See FELTON and DOUGHERTY, 163

Drinker, Cecil K. See SWIFT, HAGGART, and DRINKER, 329

Duval, Charles W., and D'Aunoy, Rigney. Studies upon experimental measles. II. The enanthematous, exanthematous, pyrexial, and leucocytic syndrome produced in the rabbit by intravenous inoculation of blood from cases of human measles, 231

Duval, Charles W., and **D'Aunoy, Rigney.** III. The symptom-complex in the guinea pig and rabbit following the intratracheal and intravenous injections of filtered nasopharyngeal secretions from cases of human measles, 239

E

Ebeling, Albert H., and **Fischer, Albert.** Mixed cultures of pure strains of fibroblasts and epithelial cells, 285

—. See CARREL and EBELING, 365, 399, 645

F

Felton, Lloyd D., and **Dougherty, Katharine M.** The organotropic, bacteriotropic, and leucocytotropic actions of certain organic chemicals, 163

Fischer, Albert. Action of antigen on fibroblasts *in vitro*. II, 535

—. Cultures of organized tissues, 393

—. A pure strain of cartilage cells *in vitro*, 379

—. See EBELING and FISCHER, 285

Flexner, Simon. Experimental epidemiology. Introductory, 9

Foot, Nathan Chandler. Studies on endothelial reactions. VI. The endothelial response in experimental tuberculous meningoencephalitis, 607

Friedrichs, Andrew V. See HARRIS and FRIEDRICHS, 219

G

Gates, Frederick L. See OLITSKY and GATES, 501, 685

H

Hagan, William A. Studies on the disease of guinea pigs due to *Bacillus abortus*, 697

—. The susceptibility of mice and rats to infection with *Bacillus abortus*, 727

—. The value of heat-killed cultures for the prevention of the *Bacillus abortus* inoculation disease of guinea pigs, 711

Haggart, Gilbert E. See SWIFT, HAGGART, and DRINKER, 329

Harris, William H., and **Friedrichs, Andrew V.** The experimental production of periarteritis nodosa in the rabbit with a consideration of the specific causal excitant, 219

Haselbauer, Peter P. See AMOSS and HASELBAUER, 107

Hess, Alfred F., Unger, Lester J., and **Pappenheimer, Alwin M.** Experimental rickets in rats. VII. The prevention of rickets by sunlight, by the rays of the mercury vapor lamp, and by the carbon arc lamp, 427

—, —, and **Steiner, Joseph M.** Experimental rickets in rats. VIII. The effect of Roentgen rays, 447

Hogue, Mary Jane. *Spirochæta eurygyrata*. A note on its life history and cultivation, 617

Howe, Paul E. See ORCUTT and HOWE, 291

Hudson, William A. The iodine content of the blood following thyroidectomy, 469

J

Jones, F. S. The source of the microorganisms in the lungs of normal animals, 317

Jones, F. S., and **Little, Ralph B.** An epidemiological study of rhinitis (coryza) in calves with special reference to pneumonia, 273

K

Krumwiede, Charles. See VALENTINE and KRUMWIEDE, 157

L

Leake, Chauncey D., and **Brown, Thomas K.** The loss of circulating erythrocytes in certain types of experimental pneumonia, 661

Lee, Ferdinand C. Changes in the number of small lymphocytes of the blood following ligation of the thoracic duct, 247

Lewis, Dean. See MAURER and LEWIS, 141

Little, Ralph B. See JONES and LITTLE, 273

—. See SMITH and LITTLE, 181, 453

Longcope, Warfield T. Insusceptibility to sensitization and anaphylactic shock, 627

Lundsgaard, Christen, and **Möller, Eggert.** Investigations on the oxygen content of cutaneous blood (so called capillary blood), 559

Lynch, Clara J. An outbreak of mouse typhoid and its attempted control by vaccination, 15

M

Maurer, Siegfried, and **Lewis, Dean.** The structure and differentiation of the specific cellular elements of the pars intermedia of the hypophysis of the domestic pig, 141

Minot, George R. Megacaryocytes in the peripheral circulation, 1

Möller, Eggert. See LUNDSGAARD and MÖLLER, 559

N

Noguchi, Hideyo. Etiology of yellow fever. XIV. Duration of the protective effect of anti-*icteroides* immune serum after subcutaneous inoculation into animals, 357

du Noüy, P. Lecomte. Surface tension of serum. III. Recovery after lowering by surface-active substances, 115

—. IV. Action of temperature, 547

O

Olitsky, Peter K., and **Gates, Frederick L.** Experimental studies of the nasopharyngeal secretions from influenza patients. IX. The recurrence of 1922, 501

— and —. X. The immunizing effects in rabbits of subcutaneous injections of killed cultures of *Bacterium pneumosintes*, 685

—. See COWDRY and OLITSKY, 521

Orcutt, Marion L., and **Howe, Paul E.** The relation between the accumulation of globulins and the appearance of agglutinins in the blood of new-born calves, 291

P

Pappenheimer, Alwin M. Experimental rickets in rats. VI. The anatomical changes which

accompany healing of experimental rat rickets, under the influence of cod liver oil or its active derivatives, 335
—. See HESS, UNGER, and PAPPENHEIMER, 427

S

Scott, W. J. M. Influence of glands with internal secretion on the respiratory exchange. IV. Effect of suprarenal insufficiency in cats, 199
Smith, Theobald, and **Little, Ralph B.** Cow serum as a substitute for colostrum in new-born calves, 453
— and —. The significance of colostrum to the new-born calf, 181
Steiner, Joseph M. See HESS, UNGER, and STEINER, 447
Storm van Leeuwen, W., Bien, Z., and **Varekamp, H.** On alimentary leucocytosis in its relation to the "*crise hémoclasique*" of Widal, 415
Swift, Homer F. The action of sodium salicylate upon the formation of immune bodies, 735
Swift, Walker E., Haggart, Gilbert E., and **Drinker, Cecil K.** A new method for measuring the pressure in the pulmonary artery, 329

T

TenBroeck, Carl, and **Bauer, Johannes H.** The tetanus bacillus as an intestinal saprophyte in man, 261

U

Unger, Lester J. See HESS, UNGER, and PAPPENHEIMER, 427
—. See HESS, UNGER, and STEINER, 447

V

Valentine, Eugenia, and **Krumwiede, Charles.** The loss of hemolytic capacity by a fraction of a culture of a hemolytic streptococcus without change in agglutination characteristics, 157
Varekamp, H. See STORM VAN LEEUWEN, BIEN, and VAREKAMP, 415

W

Webster, Leslie T. Experiments on normal and immune mice with a bacillus of mouse typhoid, 71
—. Identification of a paratyphoid-enteritidis strain associated with epizootics of mouse typhoid, 97
Wright, J. Homer, and **Craighead, Eugene M.** Infectious motor paralysis in young rabbits, 135

INDEX TO SUBJECTS.

A

Abortus:
Bacillus, disease (HAGAN) 697
—, inoculation disease, heat-killed cultures for preventing (HAGAN) 711
—, susceptibility to infection with (HAGAN) 727

Activity:
Surface tension of serum, recovery after lowering by surface-active substances (DU NOÜY) 115

Adrenal:
Insufficiency, effect on respiratory exchange (SCOTT) 199

Agglutination:
Streptococcus, hemolytic, loss of hemolytic capacity by fraction of culture without change in agglutination characteristics (VALENTINE and KRUMWIEDE) 157

Agglutinin:
Globulins and, relation of presence in blood of new-born calves (ORCUTT and HOWE) 291

Alimentary:
Leucocytosis, relation to *crise hémoclasique* (STORM VAN LEEUWEN, BIEN, and VAREKAMP) 415

Anaphylaxis:
Insusceptibility to (LONGCOPE) 627

Anatomy:
Rickets, anatomical changes accompanying healing under influence of cod liver oil (PAPPENHEIMER) 335

Antigen:
Fibroblasts *in vitro*, action on, of (FISCHER) 535
Streptococcus hæmolyticus from scarlet fever, antigenic relations between strains (BLISS) 575

Anti-icteroides:
Serum. *See* Serum.

Artery:
Pulmonary, method for measuring pressure (SWIFT, HAGGART, and DRINKER) 329

Arthritis:
Joint exudates, hydrogen ion concentration (BOOTS and CULLEN) 405

B

Bacillus:
abortus, disease (HAGAN) 697
— infection, susceptibility to (HAGAN) 727
— inoculation disease, heat-killed cultures for preventing (HAGAN) 711
Septicemia, rabbit (DE KRUIF) 309
Tetanus, as intestinal saprophyte in man (TENBROECK and BAUER) 261
Typhoid, mouse, experiments on immune mice (WEBSTER) 71
—, —, — — normal mice (WEBSTER) 71

Bacteriotropy:
Chemicals, organic (FELTON and DOUGHERTY) 163
Bacterium:
Mitochondria and, differences between (COWDRY and OLITSKY) 521
pneumosintes, immunizing effects of subcutaneous injections of killed cultures (OLITSKY and GATES) 685
Biology:
Streptococcus (BLISS) 575
Blood:
Calves, new-born, relation of presence of globulins and agglutinins (ORCUTT and HOWE) 291
Capillary, oxygen content (LUNDSGAARD and MÖLLER) 559
Changes during one day of exercise (BROUN) 481
Corpuscle, red. *See* Erythrocyte.
Cutaneous, oxygen content (LUNDSGAARD and MÖLLER) 559
Destruction during exercise (BROUN) 481
Human measles, enanthematous, exanthematous, pyrexial, and leucocytic syndrome produced by intravenous inoculation (DUVAL and D'AUNOY) 231
Iodine content after thyroidectomy (HUDSON) 469
Lymphocytes, small, changes in number following ligation of thoracic duct (LEE) 247
Body:
Immune, action of sodium salicylate on formation (SWIFT) 735
Body—*continued*:
Vaccine, supravital staining (COWDRY) 667

C

Capillary:
Blood, oxygen content (LUNDSGAARD and MÖLLER) 559
Cartilage:
Cells, pure strain *in vitro* (FISCHER) 379
Cell:
Cartilage, pure strain *in vitro* (FISCHER) 379
Epithelial, mixed cultures of pure strains of fibroblasts and (EBELING and FISCHER) 285
Pars intermedia of hypophysis, structure and differentiation of specific cellular elements (MAURER and LEWIS) 141
Chemical:
Organic, bacteriotropic actions (FELTON and DOUGHERTY) 163
—, leucocytotropic actions (FELTON and DOUGHERTY) 163
—, organotropic actions (FELTON and DOUGHERTY) 163
Circulation:
Erythrocytes, circulating, loss in pneumonia (LEAKE and BROWN) 661
Peripheral, megacaryocytes (MINOT) 1
Cod liver oil:
Rickets, anatomical changes accompanying healing under influence of (PAPPENHEIMER) 335

Colostrum:
Calf, new-born, cow serum as substitute for (SMITH and LITTLE) 453
—, —, significance to, of (SMITH and LITTLE) 181
Corpuscle:
Blood, red. *See* Erythrocyte.
Coryza:
Epidemiology in calves (JONES and LITTLE) 273
Crise hémoclasique:
Alimentary leucocytosis, relation (STORM VAN LEEUWEN, BIEN, and VAREKAMP) 415
Cultivation:
Spirochæta eurygyrata (HOGUE) 617
Culture:
Bacterium pneumosintes, killed, immunizing effects of subcutaneous injections (OLITSKY and GATES) 685
Epithelial cells and fibroblasts, mixed cultures of pure strains (EBELING and FISCHER) 285
Fibroblasts and epithelial cells, mixed cultures of pure strains (EBELING and FISCHER) 285
Heat-killed, for preventing *Bacillus abortus* inoculation disease (HAGAN) 711
Leucocytes, large mononuclear (CARREL and EBELING) 365
Organized tissues (FISCHER) 393
Streptococcus, hemolytic, loss of hemolytic capacity by fraction of culture without change in agglutination characteristics (VALENTINE and KRUMWIEDE) 157

D

Destruction:
Blood, during exercise (BROUN) 481
Disease:
Bacillus abortus (HAGAN) 697
— — inoculation, heat-killed cultures for preventing (HAGAN) 711

E

Enanthema:
Measles, human, enanthematous, exanthematous, pyrexial, and leucocytic syndrome produced by intravenous inoculation of blood from (DUVAL and D'AUNOY) 231
Endothelium:
Reaction in tuberculous meningoencephalitis (FOOT) 607
Reactions (FOOT) 607
Enteritidis:
Paratyphoid-, strain associated with epizootics of mouse typhoid (WEBSTER) 97
Epidemic:
Typhoid, mouse (LYNCH) 15
—, —, artificially induced (AMOSS) 25
—, —, control by vaccination (LYNCH) 15
—, —, spontaneous, immunological distinctions of two strains of mouse typhoid (AMOSS and HASELBAUER) 107
Epidemiology:
(AMOSS) 25, 45
(FLEXNER) 9
Coryza in calves (JONES and LITTLE) 273

Epidemiology—*continued*:
Rhinitis in calves (JONES and LITTLE) 273
Epithelium:
Cells, mixed cultures of pure strains of fibroblasts and (EBELING and FISCHER) 285
Epizootic:
Typhoid, mouse, paratyphoid-enteritidis strain associated with (WEBSTER) 97
Erythrocyte:
Circulating, loss in pneumonia (LEAKE and BROWN) 661
Etiology:
Periarteritis nodosa, specific causal excitant (HARRIS and FRIEDRICHS) 219
Yellow fever (NOGUCHI) 357
Eurygyrata:
Spirochæta (HOGUE) 617
—, cultivation (HOGUE) 617
—, life history (HOGUE) 617
Exanthema:
Measles, human, enanthematous, exanthematous, pyrexial, and leucocytic syndrome produced by intravenous inoculation of blood from (DUVAL and D'AUNOY) 231
Exercise:
Blood changes during one day of (BROUN) 481
— destruction during (BROUN) 481
Exudate:
Joint, hydrogen ion concentration in arthritis (BOOTS and CULLEN) 405
—, — — — in rheumatic fever (BOOTS and CULLEN) 405

F

Fever:
Rheumatic, hydrogen ion concentration of joint exudates (BOOTS and CULLEN) 405
Scarlet. *See* Scarlet fever.
Yellow. *See* Yellow fever.
Fibroblast:
Antigen, action *in vitro* on (FISCHER) 535
Cultures, mixed, of pure strains of epithelial cells and (EBELING and FISCHER) 285
Homologous, action of shaken serum (CARREL and EBELING) 399
Filter:
Nasopharyngeal secretions from human measles, filtered, syndrome after intratracheal injection (DUVAL and D'AUNOY) 239
— — — human measles, filtered, syndrome after intravenous injection (DUVAL and D'AUNOY) 239

G

Gland:
Internal secretion, influence on respiratory exchange (SCOTT) 199
Globulin:
Agglutinins and, relation of presence in blood of newborn calves (ORCUTT and HOWE) 291
Growth:
Leucocytes, growth-promoting function (CARREL) 385

H

Hæmolyticus:
Streptococcus, from scarlet fever, antigenic relations between strains (BLISS) 575

Healing:
Rickets, under influence of cod liver oil, anatomical changes accompanying (PAPPENHEIMER) 335
Heat:
-killed cultures for preventing *Bacillus abortus* inoculation disease (HAGAN) 711
Hemolytic:
Streptococcus, loss of hemolytic capacity by fraction of culture without change in agglutination characteristics (VALENTINE and KRUMWIEDE) 157
Homologous:
Fibroblasts, action of shaken serum (CARREL and EBELING) 399
Hydrogen ion:
Concentration of joint exudates in arthritis (BOOTS and CULLEN) 405
— — — — in rheumatic fever (BOOTS and CULLEN) 405
Hypophysis cerebri:
Pars intermedia, structure and differentiation of specific cellular elements (MAURER and LEWIS) 141

I

Immunity:
Bacillus of mouse typhoid, experiments on immune mice (WEBSTER) 71
Bodies, action of sodium salicylate on formation (SWIFT) 735
Serum, anti-*icteroides* immune, duration of protective effect after subcutaneous inoculation (NOGUCHI) 357
Immunization:
Bacterium pneumosintes, subcutaneous injections of killed cultures (OLITSKY and GATES) 685
Immunization—*continued*:
Immunology:
Typhoid, mouse, immunological distinctions of two strains (AMOSS and HASELBAUER) 107
Infection:
Bacillus abortus, susceptibility (HAGAN) 727
Paralysis, motor, infectious (WRIGHT and CRAIGHEAD) 135
Influenza:
Recurrence (OLITSKY and GATES) 501
Secretions, nasopharyngeal, from patients (OLITSKY and GATES) 501, 685
Inoculation:
Disease, *Bacillus abortus*, heat-killed cultures for preventing (HAGAN) 711
Insusceptibility:
Sensitization and anaphylactic shock (LONGCOPE) 627
Internal secretion:
See Secretion.
Intestine:
Tetanus bacillus as intestinal saprophyte in man (TENBROECK and BAUER) 261
In vitro:
Cartilage cells, pure strain (FISCHER) 379
Fibroblasts, action of antigen (FISCHER) 535
Iodine:
Blood, after thyroidectomy (HUDSON) 469

J

Joint:
Exudates, hydrogen ion concentration in arthritis (BOOTS and CULLEN) 405

Joint—*continued*:
Exudates, hydrogen ion concentration in rheumatic fever (BOOTS and CULLEN) 405

L

Lamp:
Carbon arc, prevention of rickets by (HESS, UNGER, and PAPPENHEIMER) 427
Mercury vapor, prevention of rickets by rays of (HESS, UNGER, and PAPPENHEIMER) 427

Leucocyte:
Growth-promoting function (CARREL) 385
Measles, human, enanthematous, exanthematous, pyrexial, and leucocytic syndrome produced by intravenous inoculation of blood from (DUVAL and D'AUNOY) 231
Mononuclear, large, pure cultures (CARREL and EBELING) 365
Secretions (CARREL and EBELING) 645

Leucocytosis:
Alimentary, relation to *crise hémoclasique* (STORM VAN LEEUWEN, BIEN, and VAREKAMP) 415

Leucocytotropy:
Chemicals, organic (FELTON and DOUGHERTY) 163

Ligation:
Thoracic duct, changes in number of small lymphocytes of blood following (LEE) 247

Lung:
Microorganisms, of normal animals, source (JONES) 317

Lymphocyte:
Small, of blood, changes in number following ligation of thoracic duct (LEE) 247

M

Measles:
(DUVAL and D'AUNOY) 231, 239
Human, enanthematous, exanthematous, pyrexial, and leucocytic syndrome produced by intravenous inoculation of blood from (DUVAL and D'AUNOY) 231
—, syndrome after intratracheal injection of filtered nasopharyngeal secretions from (DUVAL and D'AUNOY) 239
—, — — intravenous injection of filtered nasopharyngeal secretions from (DUVAL and D'AUNOY) 239

Megacaryocyte:
Circulation, peripheral (MINOT) 1

Meningoencephalitis:
Tuberculous, endothelial response (FOOT) 607

Method:
Pulmonary artery pressure, measurement (SWIFT, HAGGART, and DRINKER) 329

Microorganism:
Lungs of normal animals, source (JONES) 317

Mitochondria:
Bacteria and, differences between (COWDRY and OLITSKY) 521

Mononuclear:
Leucocytes, large, pure cultures (CARREL and EBELING) 365

Motor paralysis:
See Paralysis.

N

Nasopharynx:
Secretions from human measles, filtered, syndrome after intratracheal injection (DUVAL and D'AUNOY) 239
— — — —, filtered, syndrome after intravenous injection (DUVAL and D'AUNOY) 239
— — influenza patients (OLITSKY and GATES) 501, 685

O

Oil:
Cod liver. *See* Cod liver oil.
Organic:
Chemicals, bacteriotropic actions (FELTON and DOUGHERTY) 163
—, leucocytotropic actions (FELTON and DOUGHERTY) 163
—, organotropic actions (FELTON and DOUGHERTY) 163
Organism:
Cultures of organized tissues (FISCHER) 393
Organotropy:
Chemicals, organic (FELTON and DOUGHERTY) 163
Oxygen:
Blood, capillary (LUNDSGAARD and MÖLLER) 559
—, cutaneous (LUNDSGAARD and MÖLLER) 559

P

Paralysis:
Motor, infectious (WRIGHT and CRAIGHEAD) 135
Paratyphoid:
-enteritidis strain associated with epizootics of mouse typhoid (WEBSTER) 97
Pars intermedia:
Hypophysis, structure and differentiation of specific cellular elements (MAURER and LEWIS) 141
Periarteritis:
Nodosa, experimental production (HARRIS and FRIEDRICHS) 219
Pneumonia:
Epidemiological study of coryza in calves with special reference to (JONES and LITTLE) 273
— — — rhinitis in calves with special reference to (JONES and LITTLE) 273
Erythrocytes, loss of circulating (LEAKE and BROWN) 661
Pneumosintes:
Bacterium, immunizing effects of subcutaneous injections of killed cultures (OLITSKY and GATES) 685
Pressure:
Pulmonary artery, method for measurement (SWIFT, HAGGART, and DRINKER) 329
Protection:
Anti-*icteroides* immune serum, duration of protective effect after subcutaneous inoculation (NOGUCHI) 357
Pulmonary:
Artery, method for measuring pressure (SWIFT, HAGGART, and DRINKER) 329
Pyrexia:
Measles, human, enanthematous, exanthematous, pyrexial, and leucocytic syndrome

Pyrexia—*continued*:
produced by intravenous inoculation of blood from (DUVAL and D'AUNOY) 231

R

Reaction:
Endothelium (FOOT) 607
—, in tuberculous meningoencephalitis (FOOT) 607
Recovery:
Surface tension of serum, after lowering by surface-active substances (DU NOÜY) 115
Red blood corpuscle:
See Erythrocyte.
Respiration:
Glands with internal secretion, influence on respiratory exchange (SCOTT) 199
Rheumatic fever:
See Fever.
Rhinitis:
Epidemiology in calves (JONES and LITTLE) 273
Rickets:
(HESS, UNGER, and PAPPENHEIMER) 427
(HESS, UNGER, and STEINER) 447
(PAPPENHEIMER) 335
Healing under influence of cod liver oil, anatomical changes accompanying (PAPPENHEIMER) 335
Prevention by carbon arc lamp (HESS, UNGER, and PAPPENHEIMER) 427
— — rays of mercury vapor lamp (HESS, UNGER, and PAPPENHEIMER) 427
— — sunlight (HESS, UNGER, and PAPPENHEIMER) 427
Roentgen rays, effect of, on (HESS, UNGER, and STEINER) 447
Roentgen rays:
Rickets, effect on, of (HESS, UNGER, and STEINER) 447

S

Salicylate:
Sodium. *See* Sodium.
Saprophyte:
Tetanus bacillus as intestinal saprophyte in man (TENBROECK and BAUER) 261
Scarlet fever:
Antigenic relations between strains of *Streptococcus hæmolyticus* isolated from (BLISS) 575
Secretion:
Internal, influence on respiratory exchange of glands with (SCOTT) 199
Leucocytic (CARREL and EBELING) 645
Nasopharyngeal, filtered, from human measles, syndrome after intratracheal injection (DUVAL and D'AUNOY) 239
—, —, — human measles, syndrome after intravenous injection (DUVAL and D'AUNOY) 239
—, from influenza patients (OLITSKY and GATES) 501, 685
Sensitization:
Insusceptibility to (LONGCOPE) 627
Septicemia:
Bacillus, rabbit (DE KRUIF) 309
Serum:
Anti-*icteroides* immune, duration of protective effect after subcutaneous inoculation (NOGUCHI) 357

Serum—*continued*:
Cow, as substitute for colostrum in new-born calves (SMITH and LITTLE) 453
Shaken, action on homologous fibroblasts (CARREL and EBELING) 399
Surface tension (DU NOÜY) 115, 547
Shock:
Anaphylactic, insusceptibility to (LONGCOPE) 627
Skin:
Blood, cutaneous, oxygen content (LUNDSGAARD and MÖLLER) 559
Sodium:
Salicylate, action on formation of immune bodies (SWIFT) 735
Specificity:
Cellular elements of pars intermedia of hypophysis, structure and differentiation (MAURER and LEWIS) 141
Periarteritis nodosa, specific causal excitant (HARRIS and FRIEDRICHS) 219
Spirochæta:
eurygyrata (HOGUE) 617
—, cultivation (HOGUE) 617
—, life history (HOGUE) 617
Stain:
Vaccine bodies, supravital staining (COWDRY) 667
Strain:
Cartilage cells *in vitro* (FISCHER) 379
Epithelial cells and fibroblasts, mixed cultures of pure (EBELING and FISCHER) 285
Strain—*continued*:
Fibroblasts and epithelial cells, mixed cultures of pure (EBELING and FISCHER) 285
Paratyphoid-enteritidis, associated with epizootics of mouse typhoid (WEBSTER) 97
Streptococcus hæmolyticus from scarlet fever, antigenic relations (BLISS) 575
Typhoid, mouse, immunological distinctions (AMOSS and HASELBAUER) 107
Streptococcus:
Biology (BLISS) 575
hæmolyticus from scarlet fever, antigenic relations between strains (BLISS) 575
Hemolytic, loss of hemolytic capacity by fraction of culture without change in agglutination characteristics (VALENTINE and KRUMWIEDE) 157
Sunlight:
Rickets prevented by (HESS, UNGER, and PAPPENHEIMER) 427
Suprarenal:
See Adrenal.
Susceptibility:
Bacillus abortus infection (HAGAN) 727
Symptom-complex:
See Syndrome.
Syndrome:
Measles, human, enanthematous, exanthematous, pyrexial, and leucocytic syndrome produced by intravenous inoculation of blood from (DUVAL and D'AUNOY) 231
—, —, syndrome after intratracheal injection of filtered nasopharyngeal secretions from (DUVAL and D'AUNOY) 239

Syndrome—*continued*:
Measles, human, syndrome after intravenous injection of filtered nasopharyngeal secretions from (DUVAL and D'AUNOY) 239

T

Temperature:
Surface tension of serum, action on, of (DU NOÜY) 547

Tension:
Surface, of serum (DU NOÜY) 115, 547

Tetanus:
Bacillus, as intestinal saprophyte in man (TENBROECK and BAUER) 261

Thoracic duct:
Ligation, changes in number of small lymphocytes of blood following (LEE) 247

Thyroidectomy:
Iodine content of blood after (HUDSON) 469

Tissue:
Organized, cultures (FISCHER) 393

Tuberculosis:
Meningoencephalitis, endothelial response (FOOT) 607

Typhoid:
Mouse (LYNCH) 15
—, control by vaccination (LYNCH) 15
—, effect of addition of healthy mice to population suffering from (AMOSS) 45
—, epidemic artificially induced (AMOSS) 25
—, experiments on immune mice with bacillus of (WEBSTER) 71
—, — — normal mice with bacillus of (WEBSTER) 71
—, immunological distinctions of two strains (AMOSS and HASELBAUER) 107
—, paratyphoid-enteritidis strain associated with epizootics (WEBSTER) 97

V

Vaccination:
Typhoid, mouse, control by (LYNCH) 15

Vaccine:
Bodies, supravital staining (COWDRY) 667

X

X-rays:
See Roentgen rays.

Y

Yellow fever:
Etiology (NOGUCHI) 357

The Journal of General Physiology

Edited by

JACQUES LOEB, New York, N. Y.
W. J. V. OSTERHOUT, Cambridge, Mass.

The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter.

The Journal of General Physiology is issued bimonthly, one volume of about 600 pages appearing in a year. Contributions should be sent to the editors of *The Journal of General Physiology*, The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y., or 60 Buckingham Street, Cambridge, Mass. The papers should be limited preferably to twenty printed pages, not counting the space occupied by illustrations. Authors receive 100 reprints of their papers free of charge; additional copies may be obtained at cost.

The subscription price per year (one volume) is $5.00, payable in advance. Single copies cost $1.00.

Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of General Physiology*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

CONTENTS OF VOL. V, No. 2, NOVEMBER 20, 1922

De Kruif, Paul H., and Northrop, John H. The stability of bacterial suspensions. IV. The combination of antigen and antibody at different hydrogen ion concentrations.

De Kruif, Paul H., and Northrop, John H. The stability of bacterial suspensions. V. The removal of antibody from sensitized organisms.

Fenn, Wallace O. The adhesiveness of leucocytes to solid surfaces.

Fenn, Wallace O. Effect of the hydrogen ion concentration on the phagocytosis and adhesiveness of leucocytes.

Jacobs, M. H. The influence of ammonium salts on cell reaction.

Chambers, Robert. A micro injection study on the permeability of the starfish egg.

Meek, Carolyn S., and Lipman, Charles B. The relation of the reaction and of salt content of the medium on nitrifying bacteria.

Brody, Samuel, and Ragsdale, Arthur C. The equivalence of age in animals.

Harvey, E. Newton. The permeability of cells for oxygen and its significance for the theory of stimulation.

Irwin, Marian. The permeability of living cells to dyes as affected by hydrogen ion concentration.

Osterhout, W. J. V. Some aspects of selective absorption.

Loeb, Jacques. Sodium chloride and selective diffusion in living organisms.

Loeb, Jacques. The influence of salts on the rate of diffusion of acid through collodion membranes.

Northrop, John H. The mechanism of the influence of acids and alkalies on the digestion of proteins by pepsin or trypsin.

Published by

The Rockefeller Institute for Medical Research
Avenue A and 66th Street, New York, N. Y.

MONOGRAPHS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Under the head of *Monographs of The Rockefeller Institute for Medical Research* are published from time to time scientific papers which are so extensive, or require such elaborate illustrations, as to render them unsuitable for current periodical issues. The *Monographs* are published at irregular periods, determined by the available material on hand. A number of free copies, which may be 50 or less according to the size of the *Monograph*, are given to the author. *Monographs* will be sent post paid on application, payable in advance. Remittances should be made by draft or check on New York, or by postal money order, payable to The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.

No. 1. SIMON FLEXNER, J. W. JOBLING, and MAUD L. MENTEN. Experimental studies on tumor. (Issued June 30, 1910.) Price, $1.00.

No. 2. WILLIAM TRAVIS HOWARD and OSCAR T. SCHULTZ. Studies in the biology of tumor cells. Plates 1–6. (Issued February 15, 1911.) Price, $1.00.

No. 3. BENJAMIN T. TERRY. Chemo-therapeutic trypanosome studies with special reference to the immunity following cure. (Issued March 15, 1911.) Price, $1.00.

No. 4. FRANCIS W. PEABODY, GEORGE DRAPER, and A. R. DOCHEZ. A clinical study of acute poliomyelitis. Plates 1–13. (Issued June 24, 1912.) Price, $1.00.

No. 5. J. P. SIMONDS. Studies in *Bacillus welchii*, with special reference to classification and to its relation to diarrhea. (Issued September 27, 1915.) Price, $1.00.

No. 6. JAMES L. STODDARD and ELLIOTT C. CUTLER. Torula infection in man. Plates 1–9. (Issued January 31, 1916.) Price, $1.00.

No. 7. OSWALD T. AVERY, H. T. CHICKERING, RUFUS COLE, and A. R. DOCHEZ. Acute lobar pneumonia. Prevention and serum treatment. Plates 1–3. (Issued October 16, 1917.) Price, $1.00.

No. 8. ERNEST C. DICKSON. Botulism. A clinical and experimental study. Plates 1–10. (Issued July 31, 1918.) Price, $1.00.

No. 9. JAMES HOWARD BROWN. The use of blood agar for the study of streptococci. Plates 1–34. (Issued January 21, 1919.) Price, $2.00.

No. 10. WILLIAM G. MACCALLUM. The pathology of the pneumonia in the United States Army camps during the winter of 1917–18. Plates 1–53. (Issued April 16, 1919.) Price, $1.50.

No. 11. FREDERICK M. ALLEN, EDGAR STILLMAN, and REGINALD FITZ. Total dietary regulation in the treatment of diabetes. 62 charts. (Issued October 15, 1919.) Price, $4.50.

No. 12. LEWIS H. WEED, PAUL WEGEFORTH, JAMES B. AYER, and LLOYD D. FELTON. A study of experimental meningitis. A series of papers from the Army Neuro-Surgical Laboratory. Plates 1–17. (Issued March 25, 1920.) Price, $1.00.

No. 13. WERNER MARCHAND. The early stages of Tabanidæ (horse-flies). Plates 1–15. (Issued November 15, 1920.) Price, $2.00.

No. 14. S. T. DARLING and W. G. SMILLIE. Studies on hookworm infection in Brazil. First paper. (Issued February 1, 1921.) Published for the International Health Board.

No. 15. I. J. KLIGLER. Investigation on soil pollution and the relation of the various types of privies to the spread of intestinal infections. (Issued October 10, 1921.) Published for the International Health Board.

No. 16. ABRAHAM L. GARBAT. Typhoid carriers and typhoid immunity. (Issued May 10, 1922.) Price, $1.25.

No. 17. WILSON G. SMILLIE. Studies on hookworm infection in Brazil. Second paper. (Issued May 12, 1922.) Published for the International Health Board.

No. 18. P. A. LEVENE. Hexosamines, their derivatives, and mucins and mucoids. (Issued July 7, 1922.) Price, $1.25.

All inquiries, correspondence, and subscriptions should be sent to the

DIVISION OF PUBLICATION
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
AVENUE A AND 66th STREET, NEW YORK, N. Y.

CONTENTS

FOOT, NATHAN CHANDLER. Studies on endothelial reactions. VI. The endothelial response in experimental tuberculous meningoencephalitis. Plates 54 to 56.. 607

HOGUE, MARY JANE. *Spirochæta eurygyrata*. A note on its life history and cultivation. Plate 57..... 617

LONGCOPE, WARFIELD T. Insusceptibility to sensitization and anaphylactic shock..... 627

CARREL, ALEXIS, and EBELING, ALBERT H. Leucocytic secretions..... 645

LEAKE, CHAUNCEY D., and BROWN, THOMAS K. The loss of circulating erythrocytes in certain types of experimental pneumonia..... 661

COWDRY, EDMUND V. The supravital staining of vaccine bodies. Plates 58 and 59..... 667

OLITSKY, PETER K., and GATES, FREDERICK L. Experimental studies of the nasopharyngeal secretions from influenza patients. X. The immunizing effects in rabbits of subcutaneous injections of killed cultures of *Bacterium pneumosintes*. 685

HAGAN, WILLIAM A. Studies on the disease of guinea pigs due to *Bacillus abortus*. 697

HAGAN, WILLIAM A. The value of heat-killed cultures for the prevention of the *Bacillus abortus* inoculation disease of guinea pigs..... 711

HAGAN, WILLIAM A. The susceptibility of mice and rats to infection with *Bacillus abortus*..... 727

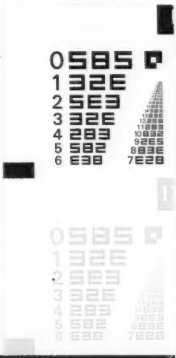
SWIFT, HOMER F. The action of sodium salicylate upon the formation of immune bodies..... 735

INDEX TO VOLUME XXXVI..... 761

The Journal of Experimental Medicine is issued monthly, two volumes appearing in a year. Each volume contains more than 600 pages and many plates and text-figures. Subscriptions will not be accepted to begin in the middle of a volume.

Contributions should be sent to the editors of *The Journal of Experimental Medicine*, Avenue A and 66th Street, New York, N. Y. They should be limited preferably to twenty printed pages, not counting the space occupied by illustrations. Articles which exceed in length twenty-five printed pages will be returned to the authors in order that their contents may be reduced to this maximum. Authors receive 100 reprints of their papers free of charge; additional copies may be obtained at cost.

The subscription price per year (two volumes) is $5.00, payable in advance. Single copies cost 75 cents. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Experimental Medicine*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

For information regarding complete sets and back numbers of *The Journal of Experimental Medicine*, application should be made to the Division of Publication of The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.

C37